10/089583



JC13 Rec'd PCT/PTO 28 MAR 2002

# APPLICATION

# FOR

# UNITED STATES LETTERS PATENT

TITLE: VACCINE

APPLICANT: JOYCE S. PLESTED, MICHAEL P. JENNINGS, MARGARET ANN J. GIDNEY, ANDREW D. COX, JAMES C. RICHARDS AND E. RICHARD MOXON

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL 940765205 US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

March 28, 2002
Date of Deposit

Signature

Leroy Jenkins
Typed or Printed Name of Person Signing Certificate

10/089583
WO 01/22994 PCT/GB00/03758
Rec'd PCT/PTO 28 MAR 2002



15/prts

# Vaccine

The present invention relates to vaccines against *Neisseria* infection, especially to infection by pathogenic *Neisseria meningitidis* and *Neisseria gonorrhoea*.

## Background of the Invention

Septicaemia and meningitis caused by *Neisseria meningitidis* remain a global health problem, especially in young children. *Neisseria meningitidis* is usually a commensal of the nasopharynx, the only major natural reservoir of this organism. The virulence factors that potentiate the capacity of *Neisseria meningitidis* to cause invasive disease include capsular polysaccharides, pili (fimbriae) or outer membrane proteins and lipopolysaccharides (DeVoe, I.W. 1982. Microbiol Rev 46: 162-190, Jennings, H.J. 1989. Contrib Microbiol Immunol 10: 151-165, Tonjum, T., and M. Koomey. 1997. Gene 192: 155-163, Nassif, X., *et al.* 1997. Gene 192: 149-153, Poolman, J.T. 1996. Adv Exp Med Biol 397: 73-33, Verheul, A.F., *et al.* 1993. Microbiol Rev 57: 34-49, Preston, A., *et al.* 1996. Crit Rev Microbiol 22: 139-180).

Existing licensed vaccines against capsular serogroups A, C, W and X are available (Frasch, C.E. 1989. Clin Microbiol Rev 2 Suppl: S134-138, Herbert, M.A., *et al.* 1995. Commun Dis Reg CDR Rev 5: R130-135, Rosenstein, N., *et al.* 1998. J.A.M.A. 279: 435-439), but generally lack satisfactory immunogenicity in very young children and do not induce long lasting protective immunity (Peltola, H., *et al.* 1977. N Engl J Med 297: 686-691, Peltola, H., *et al.* 1985. Pediatrics 76: 91-96, Reingold, A.L., C.V. Broome, *et al.* 1985. Lancet II: 114-118, Lepow, M.L., *et al.* 1986. J Infect Dis 154: 1033-1036, Cadoz, M. 1998. Vaccine

16: 1391-1395). Nonetheless, their utility has been significant in affording protection to selected populations such as the military, travellers and those at exceptional risk in outbreaks or epidemics (CDC. 1990. MMWR Morb Mortal Wkly Rep 39, No. 42: 763). Very recently, meningococcal conjugate Group C vaccines have been introduced as a routine immunisation in the United Kingdom.

The major public health priority concerning invasive meningococcal infections is to identify Group B vaccines that are highly effective in infants and give long term protection. Group B strains have accounted for a substantial, often a majority of invasive *Neisseria meningitidis* infections in many countries in Europe and North America (CDR. 1997 April. Communicable Disease Weekly Report. 7, No. 14). Prevention of Group B invasive disease represents a particularly difficult challenge in vaccine development as the capsular polysaccharide is very poorly immunogenic and even conjugates have shown disappointing immunogenicity (Jennings, H.J., and H.C. Lugowski. 1981. J. Immunology 127:1011-1018). Further, there are concerns about the safety of vaccines whose rationale is to induce antibodies to the Group B polysaccharide, a homopolymer of α-linked 2-8 neuraminic acid. The identical polysialic acid (PSA) is a post translational modification of a glycoprotein present on human cells, especially neurons, the latter is referred to as neural cell adhesion molecule (N-CAM) (Finne, J., *et al.* 1983. Lancet 2: 355-357). Both theoretical and experimental evidence have been used to argue that the induction of antibodies might result in auto-immune, pathological damage to host tissues.

Alternative approaches to develop vaccine candidates against Group B *Neisseria meningitidis* are being actively explored. These include: outer membrane porins (Poolman, J.T., *et al.* 1995. Meningococcal disease, p. 21-34K. Cartwright (ed.). John Wiley and sons, Wetzler, L.M. 1994. Ann N Y Acad Sci 730: 367-370, Rosenqvist, E., *et al.* 1995. Infect Immun 63:4642-4652, Zollinger, W.D., *et al.* 1997. Infect Immun 65: 1053-1060), transferrin binding proteins (Al'Aldeen, A.A., and K.A. Cartwright. 1996. J Infect 33: 153-157) and lipopolysaccharides (Verheul, A.F., *et al.* 1993. Infect Immun 61: 187-196, Jennings, H.J., *et al.* 1984. Infect Immun 43: 407-412, Jennings, H.J., *et al.* 1987. Antonie Van Leeuwenhoek

53: 519-522, Gu, X.X., and C.M. Tsai. 1993. Infect Immun 61: 1873-1880, Moxon, E.R., *et al.* 1998. Adv Exp Med Biol 435: 237-243).

The structure of *Neisseria meningitidis* LPS has been studied in considerable detail by Jennings H. and co-workers with additional contributions by others (Griffiss, J.M., *et al.* 1987. Infect Immun 55: 1792-1800, Stephens, D.S., *et al.* 1994. Infect Immun 62: 2947-2952, Apicella, M.A., *et al.* 1994. Methods Enzymol 235: 242-252, Poolman, J.T. 1990. Polysaccharides and membrane vaccines, p.57-86. In Bacterial vaccines, A. Mizrahi (ed.)., *et al.* 1997. FEMS Microbiol Lett 146: 247-253). The structures of major glycoforms for several immunotypes (L1-L9) have been published L1, L6 (Di Fabio, J.L., *et al.* 1990. Can J Chem 68: 1029-1034, Wakarchuk, W.W., *et al.* 1998. Eur J Biochem 254: 626-633); L3 (Pavliak, V., *et al.* 1993. J Biol Chem 268: 14146-14152); L5 (Michon, F., *et al.* 1990. J. Biol Chem 265:7243-7247); L2 (Gamian, A., *et al.* 1992. J Biol Chem 267: 922-925); L4,L7 (Kogan, G., *et al.* 1997. Carbohydr Res 298: 191-199): L8 (Wakarchuk, W.W., *et al.*, 1996, J. Biol. Chem. 271, 19166 - 19173), L9 (Jennings, H.J., *et al.* 1983. Carbohydr. Res. 121: 233-241). Reference is also made to the following discussion of the accompanying Figure 1.

It is known that, in addition to this inter-strain variation, individual *Neisseria meningitidis* strains exhibit extensive phase variation of outer core LPS structures (reviewed in van Putten, J.P., and B.D. Robertson. 1995.Mol Microbiol 16: 847-853 and Andersen, S.R., *et al.* 1997. Microb Pathog 23: 139-155). The molecular mechanism of this intra strain variation involves hypermutable loci within the reading frames encoding several glycosyl transferases (Gotschlich, E.C. 1994. J Expt Med 180: 2181-2190, Jennings, M.P., *et al.* 1995. Mol Microbiol 18: 729-740). Similar mechanisms of phenotypic variation have been reported for other phase-variable surface components of pathogenic *Neisseria*, including Opc (Sakari, J., *et al.* 1994. Mol. Microbiol 13: 207-217), Opa (Stern, A., *et al.* 1986. Cell 47: 61-71) and PilC proteins (Jonsson, A.B., *et al.* 1991. EMBO J 10: 477-488). The high frequency, reversible molecular switching is mediated by homopolymeric tracts of cytosines or guanines through slippage-like mechanisms that results in frame shifts (Gotschlich, E.C. 1994. J Expt Med 180: 2181-2190, Jennings, M.P., *et al.* 1995. Mol Microbiol 18: 729-740, Stern, A., and T.F. Meyer. 1987. Mol. Microbiol 1: 5-12).

Despite the extensive antigenic variation of LPS, the inner core of the LPS has been considered to be relatively highly conserved, and therefore the use of the inner core of the LPS structure has been suggested for use in vaccine design. However, the problems with candidate vaccine generation in this way are numerous.

First, although it was known that certain components of the inner core could be immunogenic (Jennings, H.J. Lugowski, C. and Ashton, F.E. 1984. Infect. Immun. 43: 407-412 and Verheul, A.F., *et al.*, 1991. Infect. Immun. 59: 3566-3573), the extent of conservation of these epitopes across the diversity of meningococcal disease isolates was not known and evidence of bactericidal activity of antibodies to these epitopes has not been shown. US-A-5,705,161 discloses that oligosaccharides of meningococcal immunotypes differ, for example, with regard to monosaccharide composition, amount and location of phosphoethanolamine groups and degree of acetylation of the inner core GlcNAc unit or other units, indicating that many possible structures may be found in the core structure. US-A-5,705,161 also suggests that a portion of the core of a meningococcal LPS may be suitable for use in a vaccine, although no specific immunogenic epitopes or supporting data are disclosed.

Secondly, given the presence of the outer core LPS structure and other surface exposed non-LPS structures, including capsule, it is not known whether the inner core structure is accessible to the immune system to allow a bactericidal immune response to be generated. Furthermore, any vaccine would need to contain immunogenic structures which elicit an immune response to the complete range of pathogenic *Neisseria meningitidis* strains. However, the extent of variation exhibited by the inner core structure of virulent strains is not known, and rigorous investigation of the problem has not been undertaken.

Furthermore, in the publication New Generation Vaccines [1997, Ed. M. M. Levine, publ. Marcel Deker Inc, New York, Chapter 34, page 481], it is stated that, with respect to vaccine development, 'including LPS that consists only of the common inner core region of the oligosaccharide may not result in induction of bactericidal antibodies...".

In addition, other species of the genus *Neisseria* pose global health problems. For example, *Neisseria gonorrhoeae* is involved in sexually transmitted diseases such as urethritis, salpingitis, cervicitis, proctitis and pharyngitis, and is a major cause of pelvic inflammatory disease in women.

Accordingly, there is still a need in the art for an effective vaccine against pathogenic *Neisseria* infection, such as *Neisseria meningitidis* and *Neisseria gonorrhoeae* infection.

The present invention sets out to address this need.

**Statement of invention**

In a first aspect, the invention relates to a vaccine for the treatment of disease caused by *Neisseria* infection, the vaccine comprising an immunogenic component of *Neisseria* strains. The vaccine presents a conserved and accessible epitope that in turn promotes a functional and protective response.

We have now discovered that the inner core of the LPS of *Neisseria* can be used to generate a protective immune response to *Neisseria* infections, for example *Neisseria meningitidis* infections. For simplicity the present invention is herein exemplified principally by discussion of vaccines and treatments against *Neisseria meningitidis* infections, but the invention extends to diseases caused by other pathogenic *Neisseria* species.

Using a range of novel monoclonal antibodies, epitopes belonging to the inner core of *Neisseria meningitidis* have been identified which have been found to be accessible to the immune system, and which are capable of stimulating the production of functional, protective antibodies. Moreover, analysis of *Neisseria meningitidis* strains using the new antibody tools indicates that certain epitopes are common to a range of *Neisseria meningitidis* disease isolates, and sometimes occur in a majority of such strains. Accordingly a vaccine comprising only a limited range of *Neisseria meningitidis* inner core epitopes can provide

effective immunoprophylaxis against the complete range of strains causing *Neisseria meningitidis* infection. Similar considerations apply to other pathogenic species.

In a related aspect, the invention provides a vaccine effective against strains of the bacteria of the genus *Neisseria*, especially strains of the species *Neisseria meningitidis*. Particularly in the latter instance, the vaccine comprises one or more immunogens which can generate antibodies that recognise epitopes in encapsulated strains. The one or more immunogens represent one or more accessible inner core epitopes. Thus, the immunogens can give rise to antibodies that recognise a majority of strains.

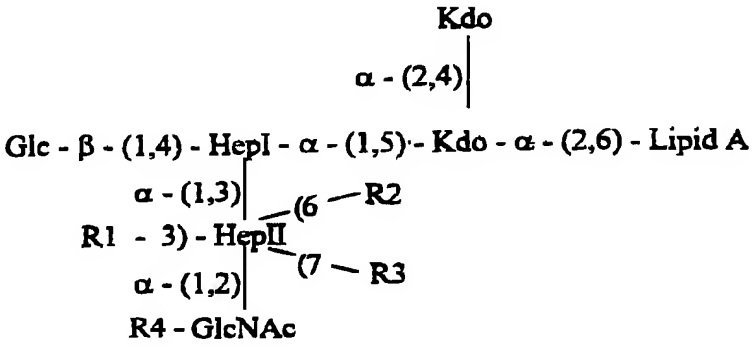
We use the word "principal" to refer to a majority. Thus, a principal immunogenic component elicits antibodies to a majority of strains.

In our approach, antibodies were generated by immunising mice using *Neisseria meningitidis galE* mutants. The antibodies produced were specific to the LPS inner core because *galE* mutants lack outer core structures. The reactivity of these antibodies against a panel of *Neisseria meningitidis* strains representative of the diversity found in natural populations of disease isolates was investigated. One monoclonal antibody reacted with 70 % of all *Neisseria meningitidis* strains tested, suggesting strong conservation of the inner core epitope recognised by this antibody, termed antibody B5. The epitope against which B5 reacts has been characterised and can be used to form the basis of a vaccine to prevent *Neisseria* infections.

A hybridoma producing the monoclonal antibody B5, designated hybridoma NmL3B5, has been deposited under the Budapest Treaty on 26 September 2000 with the International Depositary Authority of Canada in Winnipeg, Canada, and given the Accession Number IDAC 260900-1.

In this way, we have obtained proof in principle that one or more of the inner core epitopes of LPS are conserved and accessible to antibodies, that a specific immune response to these epitopes can mediate protection, and that LPS inner core oligosaccharides can be candidate

vaccines. The inner core LPS typically consists of an inner core oligosaccharide attached to lipid A, with the general formula as shown:




where R1 is a substituent at the 3-position of HepII, and is hydrogen or Glc-α-(1, or phosphoethanolamine; R2 is a substituent at the 6-position of HepII, and is hydrogen or phosphoethanolamine; R3 is a substituent at the 7-position of HepII, and is hydrogen or phosphoethanolamine, and R4 is acetyl or hydrogen at the 3-postion, 4-position or 6-position of the GlcNAc residue, or any combination thereof; and where Glc is D-glucopyranose; Kdo is 3-deoxy-D-*manno*-2-octulosonic acid; Hep is L-*glycero*-D-*manno*-heptose, and GlcNAc is 2-acetamido-2-deoxy-D-glucopyranose.

**General Description of the Invention**

The principal immunogenic component for *Neisseria meningitidis* strains is preferably a single immunogenic component found in at least 50 % of *Neisseria meningitidis* strains, i.e. in the majority of naturally occurring *Neisseria meningitidis* strains. The principal immunogenic component forms a candidate vaccine immunogen. Preferably the immunogenic component of the vaccine of the present invention is any one element or structure of *Neisseria meningitidis* or other species of *Neisseria* capable of provoking an immune response, either alone or in combination with another agent such as a carrier. Preferably the principal immunogenic component comprises of or consists of an epitope which is a part or all of the inner core structure of the *Neisseria meningitidis* LPS. The immunogenic component may also be derived from this inner core, be a synthetic version of the inner core, or be a functional equivalent thereof such as a peptide mimic. The inner core

LPS structure of *Neisseria meningitidis* is generally defined as that shown in Fig 1 and as outlined in the figure legend. The immunogenic component is suitably one which elicits an immune response in the presence and in the absence of outer core LPS.

The principal immunogenic component is conserved in at least 50% of *Neisseria* strains within the species, preferably at least 60%, and more preferably at least 70%. Reactivity with 100% strains is an idealised target, and so the immunogenic component typically recognises at most 95 %, or 85 % of the strains. Conservation is suitably assessed functionally, in terms of antibody cross-reactivity. We prefer that the immunogenic component is present in at least 50 % of serogroup B strains, preferably at least 60%, more preferably at least 70%, even more preferably at least 76%. Suitably, assessment of the cross reactivity of the immunogenic component is made using a representative collection of strains, such those outlined in Maiden [Maiden M.C.J., *et al.*, 1998, P.N.A.S.95, 3140 - 3145].

Preferably the principal immunogenic component is found in the *Neisseria meningitidis* immunotype L3, and preferably it is not in L2. More specifically, we prefer that the immunogen is found in the immunotypes L1, L3, L7, L8 and L9, but not in L2, L4, L5 or L6. In other words, we prefer that the immunogen, notably the principal immunogenic component, generates antibodies which are reactive with at least the L3 immunotype, and usually the L1, L3, L7, L8 and L9 immunotypes, but not with L2, and usually not the L2, L4, L5 and L6 immunotypes. There are conformational differences forced on the inner core of the L2 and L3 immunotypes by different arrangements at HepII, namely the PEtn moiety at the 6-position in L2 or at the 3-position in L3, and the Glc residue at the 3-postion in L2. Currently we do not envisage the possibility of a single epitope for both L2 and L3 immunotypes. In other words, without dismissing the possibility of a single epitope, the present invention is expected to require different immunogens to elicit antibodies for L2 and L3.

Preferably the principal immunogenic component is a conserved epitope on the LPS inner core recognised by an antibody termed B5 herein. The preferred epitope of the invention is thus any epitope recognised by the B5 antibody.

Preferably the immunogenic component is a conserved epitope on the LPS inner core defined by the presence of a phosphoethanolamine moiety (PEtn) linked to the 3-position of HepII, the β-chain heptose, of the inner core, or is a functional equivalent thereof. In this respect, where the context permits, HepI and HepII refer to the heptose residues of the inner core oligosaccharide which respectively are proximal and distal to the lipid A moiety of the neisserial LPS, without being necessarily tied to the general formula given above.

Preferably this epitope comprises a glucose residue on HepI, the α-chain heptose residue. While this glucose is not necessary for B5 binding, it is required for optimal recognition.

The principal immunogenic component of the present invention is preferably an epitope on the LPS inner core which comprises an N-acetyl glucosamine on HepII. The presence of N-acetyl glucosamine is required for optimal recognition by B5.

Preferably the principal immunogenic component comprises both the N-acetyl glucosamine on HepII and a glucose residue on HepI.

The immunogenic component of the present invention is typically only limited by the requirement for a phosphoethanolamine moiety (PEtn) linked to the 3-position of HepII of the inner core, which is required for B5 reactivity. The structure of the inner core may be modified, replaced, or removed, as necessary, to the extent that these are not needed. Similarly, any outer core structures may be modified or deleted, to the extent that structural elements are not needed. There is no requirement for the immunogenic component to lack the outer core portion, or equivalent, of the LPS. The immunogenic component may comprise outer core elements having a galactose component, for example the terminal galactose residue of the lacto-N-neotetraose. In one suitably embodiment, the immunogenic component is derived from LPS and is free from other cellular material. Alternatively, cellular material may be present, and can take the form of live or killed bacteria.

In a related aspect, the vaccine of this invention has an immunogenic epitope recognised by an antibody to a *galE* mutant of *Neisseria meningitidis*.

In a further embodiment the vaccine suitably comprises further immunogenic elements from the inner core with an aim to achieving up to 100% coverage. Preferably the vaccine comprises only a limited number (4-6, or less) of immunogenic elements, more preferably only those glycoforms which are representative of all possible PEtn positions on HepII, the β-chain heptose, of the inner core, i.e. wherein PEtn is at the 3-position, exocyclic (6-position or 7-position) or absent, with or without an α-1-3 linked glucose at HepII, or a combination thereof. The presence of PEtn substituent is not required for the generation of antibodies by an immunogenic component of this invention.

Moreover, as detailed herein, the epitopes of this invention are immunogenic and accessible, and thus can be used to develop an effective vaccine. Furthermore, as detailed herein, a vaccine containing only a limited number of glycoforms (representing all the possible PEtn positions on HepII, namely position 3-, or 6-, or 7- or none, and combinations thereof), is able to effectively provide protection against the diverse range of meningococcal isolates causing invasive disease.

Accordingly the vaccine of the present invention preferably comprises an epitope which is defined by the presence of a phosphoethanolamine moiety (PEtn) linked to the 3-position of HepII of the inner core, and additionally comprises an epitope defined by the presence of PEtn on the 6-position of HepII of the inner core, and/or an epitope defined by PEtn on the 7-position of HepII of the inner core, or wherein there is no additional PEtn addition. Preferably the vaccine contains only immunogenic components which are these inner core glycoform variants.

The B5 antibody of the present invention also recognises the inner core structures of *Neisseria gonorrhoeae* and *Neisseria lactamica*. As such, the invention extends to any *Neisseria* species, and any reference to *Neisseria meningitidis* can as appropriate be extended to other *Neisseria* species, preferably *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Neisseria lactamica*, most preferably *Neisseria meningitidis*. The invention also extends to immunogenic components in other *Neisseria* species which are related to those identified in

*Neisseria meningitidis*, either by function, antibody reactivity or structure. The invention is not limited to pathogenic strains of *Neisseria*. The vaccine of this invention can be derived from a commensal strain of Neisseria, especially a strain of *Neisseria* lactamica. The species *Neisseria lactamica* is typically strongly immunogenic, and therefore we prefer that the LPS inner core immunogenic component is derived from this species.

The vaccine may thus be homologous or heterologous, and thus founded on an immunogenic component from the target micro-organism, homologous, or from a different micro-organism, heterologous. The micro-organism can be naturally occurring or not, such as can be produced by recombinant techniques. In particular, the micro-organism can be engineered to modify the epitope or to modify other components.

In a further aspect of the invention we have determined that a second monoclonal antibody, herein termed A4, is able to react with inner core epitopes of nearly all of the *Neisseria meningitidis* strains which do not react with the B5 antibody. Thus, of the 100 *Neisseria meningitidis* strains tested, 30% were not reactive with B5 and were found to lack a PEtn moiety at the 3-position of HepII. Of these 30 strains, 27 were reactive with A4. Accordingly, a vaccine comprising only 2 inner core epitopes, corresponding to those epitopes defined by cross reactivity with A4 and B5, provides 97% coverage of a representative collection of *Neisseria meningitidis* strains, preferably as assessed by using the collection of strains as outlined in Maiden *et al.* [*supra*]. A preferred epitope of the invention is thus also any epitope recognised by the A4 antibody.

A hybridoma producing the monoclonal antibody A4, designated hybridoma NmL4galEA4, has been deposited under the Budapest Treaty on 26 September 2000 with the International Depositary Authority of Canada in Winnipeg, Canada, and given the Accession Number IDAC 260900-2.

The present invention thus also relates to a vaccine comprising a few immunogenic components, wherein at least 70% of *Neisseria meningitidis* strains of the species possess at least one of the immunogenic components, preferably 80%, preferably 90%, and most

preferably 97%. In this way the vaccine can give protective coverage against *Neisseria* infection in 70%, preferably 80%, 90% or even 97% or more of cases.

A few immunogenic components suitably means at least two immunogenic components, preferably only 2. More generally the few components comprise 2 to 6 components, such as 2, 3, 4, 5 or 6 components, more suitably 2, 3 or 4 components. Preferably the immunogenic components are a few glycoforms of the inner core, representative of all natural *Neisseria meningitidis* strains. In this way, a vaccine containing a limited number of glycoforms can give approaching 100% coverage of *Neisseria meningitidis* strains.

A representation of the 3D structures of the LPS inner core having a PEtn moiety at the 3-position, 6-position or absent at HepII are shown in Fig. 3. Accordingly, the present invention also extends to immunogenic elements which have the same or similar structures to these inner core structures, as defined by their 3D geometry and to antibodies capable of interacting with such structures, either as assessed *in vitro*, *in vivo* or *in silico*.

The immunogenic elements of the invention are preferably those shown to elicit antibodies having opsonic and bactericidal activity, and shown to generate antibodies which confer passive protection in *in vivo* models.

The invention also extends to use of any immunogenic element as defined above in the preparation of a medicament for the prevention, treatment or diagnosis of *Neisseria* infection.

The candidate vaccine immunogens of the present invention may be suited for the prevention of all *Neisseria* infections. However, a vaccine for the treatment of *Neisseria meningitidis* is preferred, with a vaccine for group B strains especially preferred.

Preferably the immunogenic element of the vaccine is accessible in the presence of bacterial capsule. Accordingly, antibodies generated by an individual who is vaccinated will be able to access the same epitope on invading strains of *Neisseria*, and thus protect the individual from

infection. Antibodies given directly to a patient for treatment, also are thus able to directly access the target *Neisseria* strains.

Preferably the vaccine of the present invention comprises epitopes which are capable of stimulating antibodies which are opsonic. We further prefer that these antibodies are capable of binding to wild type *Neisseria* strains to confer protection against infection and which are bactericidal.

The present invention also provides a method for treating pathogenic *Neisseria*. The method employs one or a few immunogenic components which give rise to effective antibodies, and which rely on an inner core epitope for stimulating the immune response. The immune response is ordinarily B cell mediated, but we can include T cell mediated immunity. The antibodies generated by the vaccine of this invention bind to inner core elements of the pathogenic target bacterium.

Diseases caused by *Neisseria meningitidis* include principally meningitis, septicaemia and pneumonia, and the prevention and treatment of these diseases is especially preferred in the present invention. Diseases caused by *Neisseria gonorrhoeae* include sexually transmitted diseases such as urethritis, cervicitis, proctitis pharyngitis, salpingitis, epididymitis and bacteremia/arthritis. Additionally, the invention extends to treatment and prevention of any other disease which results from *Neisseria* infection, especially to diseases in which *Neisseria* infection could weaken the immune system such that another disease or pathogen could be harmful to an individual. The treatment can be preventative or curative.

The vaccine of the present invention is a formulation suitable for safe delivery to a subject, allowing the subject to develop an immune response to future infection by *Neisseria*. Vaccines of the present invention are preferably formulated vaccines in which any of the immunogenic components of the vaccine may be conjugated, and any suitable agent for conjugation may be used. Conjugation enables modification of the presentation of the antigen, and may be achieved by conventional techniques. Examples of agents for conjugation include proteins from homologous or heterologous species. In this way, the

immunogenic component of the present invention forms a saccharide peptide conjugate. Preferably the peptide portion comprises a T cell activating epitope.

The vaccines of the present invention may be delivered with an adjuvant, to enhance the immune response to the immunogenic components. Suitable adjuvants include aluminium salts, oils in combination with bacterial macromolecules, liposomes, muramyl dipeptide. ISCOMS, bacterial toxins such as pertussis, cholera and those derived from *E. coli* and cytokines such as IL-1, IL-2 and IFNγ.

The vaccine of the invention may be delivered by suitable means, such as by oral delivery or parenteral administration, injection, nutraceutical or other delivery means, and may be provided in any suitable delivery form such as tablets, pills, capsules granules, solutions, suspensions or emulsions. Suitably the vaccine components are prepared in the form of a sterile, isotonic solution.

The present invention also extends to the monoclonal antibodies derived from the concepts and methodologies described herein, including but not limited to B5 and A4, and use of these antibodies in the treatment of *Neisseria* infection. The invention also relates to pharmaceutical preparations comprising such antibodies in combination with a pharmaceutically acceptable carrier. Such preparations may be delivered by any suitable means, such as those exemplified above for vaccine delivery, and used in combination with other active agents or adjuvants.

The correct dosage of the antibody or vaccine will vary according to the particular formulation, mode of application, and the particular host being treated. Factors such as age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, and reaction sensitivities are suitably to be taken into account.

The antibodies and vaccines compositions of the present invention may be used with other drugs to provide combination treatments. The other drugs may form part of the same

composition, or be provided as a separate composition for administration at the same time or a different time.

In addition to the antibodies themselves, the invention also relates to the hybridomas which produce such antibodies.

Antibodies against the immunogenic components of the invention may be generated by administering the immunogenic components to an animal, preferably a non-human animal, using standard protocols. For the preparation of monoclonal antibodies, any suitable techniques can be used. Techniques for the production of single chain antibodies (US 4,946,778) can be adapted to produce appropriate single chain antibodies. Moreover, transgenic mice or other organisms or animal may be used to express humanised antibodies immunospecific to the immunogenic components of the invention.

Alternatively, other methods, for example phage display technology may be used to select antibody genes for proteins with binding activities towards immunogenic components of the present invention.

Antibodies of the invention may be either monoclonal or polyclonal antibodies, as appropriate.

The present invention also relates to a method for the prevention of *Neisseria* infection, the method comprising administering to a subject in need of such treatment an effective amount of a vaccine as described above.

Preferably the administration is adequate to produce a long lasting antibody and/or T cell immune response to protect the subject from infection, particularly *Neisseria meningitidis* infection.

The invention also relates to a method for the treatment of *Neisseria* infection, the method comprising administering to a subject in need of such treatment an effective amount of an

antibody to the *Neisseria meningitidis* inner core. Preferably, the antibody is B5 or A4. or an antibody which recognises the same epitope as B5 or A4, or an antibody derived from the concepts and methodologies herein described, or is a combination thereof.

Moreover, the methods of the invention may be extended to identification of epitopes in any bacterial strain. Epitopes so identified may be tested both for accessibility, conservation across the population and functional activity, using methods as outlined in the attached Examples. The present invention thus additionally relates to a method for the identification of an immunogenic element, comprising raising an antibody to a bacterial structure, preferably a bacterial LPS structure, more preferably a bacterial inner core LPS structure, and testing the epitope recognised by the antibody for accessibility to antibody in the wild type strain, optionally also comprising testing the epitope for conservation across the bacterial population and testing for functional activity to the epitope *in vivo*.

Preferably the bacterial species are *Neisseria* species, preferably *Neisseria meningitidis*, *Neisseria gonorrhoeae* or *Neisseria lactamica*.

Specifically, the present invention provides a method to generate antibodies to the inner core of *Neisseria meningitidis*. For the first time it has been possible to screen a population of *Neisseria meningitidis* strains to identify whole population features which are independent of immunotype.

Accordingly, the present invention also relates to a method for the identification of immunogenic epitopes of *Neisseria meningitidis*, the method comprising the steps of:

1 generating antibodies to the inner core of *Neisseria meningitidis*, by inoculation of a host organism with a *galE* mutant strain of *Neisseria meningitidis*, and

2 testing such antibodies against a wild type *Neisseria meningitidis* strain to identify those antibodies which are reactive, and for which the epitopes are therefore accessible.

The potential utility of epitopes so identified may be further assessed by screening antibodies which react with the inner core of *Neisseria meningitidis galE* strain against a panel of strains which are representative of strain diversity. Preferably the strain panel is selected using an approach based upon a population analysis. Epitopes so identified may then be tested in functional assays, as outlined in Example 3.

In particular the invention extends to a method for the analysis of antibody binding to bacteria, wherein natural isolates of bacteria are studied when grown on and adherent to tissue cultured cells, such as HUVECs. This assay provides a monolayer of cells to which the bacteria adhere in a biologically relevant environment. Previous attempts using Neisseria, for example, directly adherent to gelatin- or matrigel-coated coverslips resulted in low numbers of adherent bacteria after repeated washings and high non-specific background staining. In particular we prefer that the antibody binding is analysed using confocal microscopy.

This method also identifies antibodies suitable for therapeutic use, and the invention extends to such antibodies.

Moreover, key biosynthetic genes for each step in LPS synthesis have been identified (Preston *et al.*, 1996, Crit. Rev. Microbiol. 22, 139 - 180) and this allows the construction of a series of mutants from which LPS glycoforms of varying size and complexities can be made available to facilitate the identification of conserved epitopes (van der Ley *et al.*, 1997, FEMS Microbiol. Letter 146, 247 - 253, Jennings et al 1993, Mol. Microbiol. 10 361 - 369, Jennings *et al.*, 1995, Microb Pathog 19, 391 - 407, van der Ley *et al.*, 1996, Mol Microbiol 19, 1117 - 1125).

The present invention also relates to the gene found in *Neisseria meningitidis* which is involved in PEtn substitution at the 3- position on HepII, and to genes related in structure and function. As yet no genes have been identified in any bacteria that are involved in addition of PEtn to LPS structures. Using B5, specific for an inner core LPS epitope containing a PEtn, we have identified a putative LPS phosphoethanolamine transferase gene (designated *hypo3*) in *Neisseria meningitidis*. *Hypo3* was named arbitrarily by us, as it is the 3rd reading frame

in a fragment of DNA selected by experimentation, from the MC58 genome sequence. The original *hypo3* is therefore from MC58. This ORF is called NMB2010 in the TIGR data base (MC58 genome sequence) and although designated as a protein of unknown function, they classify it as a "YhbX/YhjW/YijP/YjdB family protein". This indicates that homologues have been inferred in other organisms but they do not know the function of them. The homologue in the serogroup A sequence at the Sanger Centre is designated NMA0431, although this gene is smaller than *hypo3*. *Hypo3* is involved in PEtn substitution at the 3-position at HepII. Furthermore, the presence of the complete gene is required for the expression of the B5 reactive phenotype in *Neisseria meningitidis* as well as other pathogenic and commensal *Neisseria* species.

The identification of the gene allows mutants to be created which are isogenic apart from *hypo3*, and which differ only in the presence or absence of PEtn at the 3-position of HepII in the LPS inner core. Such strains can be used in comparative studies. Moreover, strains appropriate for vaccine production can be engineered so that they comprise the preferred PEtn structure at the 3-position, or engineered so that this PEtn cannot be present.

Accordingly, the invention relates to use of the *hypo3* gene, or homologue thereof, in the production of an *Neisseria* strain for the assessment, treatment or prevention of *Neisseria* infection. The homologue may have 60%, 70%, 80%, 90% or more homology or identity to *hypo3*, as assessed at the DNA level. Use of the gene includes the methods outlined above, for preparing genetically modified strains for vaccination, isolation of appropriate epitopes and generation of strains for comparative studies. More generally, we envisage the identification and use of any gene which plays a role in the biosynthetic pathway, and which has an effect on the conservation, accessibility or function of the immunogen.

The present invention is now illustrated by the following Figures and Examples which are not limiting upon the present invention, wherein:

Figure 1 illustrates the LPS structure of various *Neisseria meningitidis* immunotypes;

Figure 2 illustrates cross reactivity of B5 with selected immunotypes and mutants of *Neisseria meningitidis* LPS;

Figure 3 illustrates molecular models of the calculated (MMC) lowest energy states of the core oligosaccharide from *galE* mutants of L3, L4 and L8 dephosphorylated;

Figure 4 illustrates cross reactivity of B5 with genetically modified L3 LPS and chemically modified L8 LPS from *Neisseria meningitidis*;

Figure 5 illustrates confocal immunofluorescence microscopy of *Neisseria meningitidis* organisms strain MC58 adherent to HUVECs;

Figure 6 illustrates silver stained tricine gels of LPS preparations from *Neisseria meningitidis* group B strains not reactive with B5;

Figure 7 illustrates accessibility of the LPS epitope to A4 in *Neisseria meningitidis* whole cells;

Figure 8 illustrates conservation of the LPS epitope across *Neisseria meningitidis* serogroups;

Figure 9 illustrates the strategy for the Example 2;

Figure 10 illustrates ELISA titres of antibodies to L3 *galE* LPS (IgG) in paired sera taken early and late from children with invasive meningococcal disease, and mean % phagocytosis of *Neisseria meningitidis* MC58 with paired sera taken early and late from children with invasive meningococcal disease with human peripheral blood mononuclear cells and human complement;

Figure 11a illustrates mean % phagocytosis of *Neisseria meningitidis* MC58 with MAb B5 pre-incubated with increasing concentrations of either (i) B5 reactive or (ii) B5 non-reactive *galE* LPS with human peripheral blood polymorphonuclear cells and human complement;

Figure 11b illustrates mean % phagocytosis of pair of *Neisseria meningitidis* wild-type isogenic strains (*Neisseria meningitidis* BZ157) that are either MAb B5 reactive or B5 non-reactive with MAb B5 as the opsonin with human peripheral blood mononuclear cells and human complement;

Figure 11c illustrates mean % phagocytosis of fluorescent latex beads coated with either purified LPS from L3 *galE* mutant (10μg/ml) or uncoated, in the presence of MAb B5 or final buffer, with human peripheral blood mononuclear cells and human complement;

Figure 12 illustrates mean % survival of *Neisseria meningitidis galE* mutant in the presence and absence of MAb B5 against two-fold serial dilutions of human pooled serum starting at 40% as detected using a serum bactericidal assay;

Figure 13 illustrates Geometric mean bacteremia in the blood of groups of 5 day old infant rats 24h post-infection with 1 x $10^8$ cfu/ml *galE* mutant given simultaneously with: (i) no antibody; (ii) MAb B5 (10μg dose); (iii) MAb B5 (100μg dose); or (iv) MAb 735, a positive control anti-capsular antibody (2μg dose);

Figure 14 illustrates a Western blot showing purified LPS from *Neisseria meningitidis* MC58 and *galE* mutant probed with MAb B5 (ascites fluid 1:2000) detected using anti-mouse IgG alkaline phosphatase and BCIP/NBT substrate; and

Figure 15 illustrates a FACS profile comparing surface labelling of live *Neisseria meningitidis* MC58 and *galE* mutant (5 x 108 org./ml) with MAb B5 (culture supernatant 1:50) detected using anti-mouse IgG (FITC labelled).

**Examples of the Invention**

**Example 1 Identification of immunogenic epitopes in *Neisseria meningitidis***

**Introduction**

We investigated the conservation and antibody accessibility of inner core epitopes of *Neisseria meningitidis* lipopolysaccharide (LPS) because of their potential as vaccine candidates. An IgG3 murine monoclonal antibody (MAb), designated MAb B5, was obtained by immunising mice with a *galE* mutant of *Neisseria meningitidis* H44/76 (B.15.P.1.7.16 immunotype L3). We have shown that MAb B5 can bind to the core LPS of wild-type encapsulated MC58 (B.15.P1.7.16 immunotype L3) organisms *in vitro* and *ex-vivo*. An inner core structure recognised by MAb B5 is conserved and accessible in 26/34 (76%) of Group B and 78/112 (70%) of Groups A,C,W,X,Y and Z strains. *Neisseria meningitidis* strains which possess this epitope are immunotypes in which phosphoethanolamine (PEtn) is linked to the 3-position of the β-chain heptose (HepII) of the inner core. In contrast, *Neisseria meningitidis* strains lacking reactivity with MAb B5 have an alternative core structure in which PEtn is linked to an exocyclic position (i.e. position 6 or 7) of HepII (immunotypes L2, L4 and L6) or is absent (immunotype L5). We conclude that MAb B5 defines one or more of the major inner core glycoforms of *Neisseria meningitidis* LPS.

These findings encourage the possibility that immunogens capable of eliciting functional antibodies specific to inner core structures could be the basis of a vaccine against invasive infections caused by *Neisseria meningitidis*.

In summary, we report that a monoclonal antibody, designated B5, has identified a cross-reacting epitope on the LPS of the majority of naturally occurring, but genetically diverse strains of *Neisseria meningitidis*. Critical to the epitope of strains recognised by the monoclonal antibody B5 is a phosphoethanolamine (PEtn) on the 3-position of the β-chain heptose (HepII) (Figure 1). In contrast, all *Neisseria meningitidis* strains lacking reactivity with MAb B5 are immunotypes characterised by the absence of PEtn substitution or by PEtn substitution at an exocyclic position (i.e. position 6 or 7) of HepII (Figure 1). Thus, a limited repertoire of inner core LPS variants is found among natural isolates of *Neisseria meningitidis*

strains and these findings encourage the possibility that a vaccine might be developed containing a few glycoforms representative of all natural *Neisseria meningitidis* strains.

## Materials and Methods

### Bacterial strains

The *Neisseria meningitidis* strains MC58 and H44/76 (both B:15:P1.7.16 immunotype L3) have been described previously (Virji, M., H. Kayhty, D.J.P. Ferguson, J.E. Heckels, and E.R. Moxon, 1991. Mol Microbiol 5: 1831-1841, Holten, E. 1979. J Clin Microbiol 9: 186-188). Derivatives of MC58 and H44/76 with defined alterations in LPS were obtained by inactivating the genes, *galE* (Jennings, M.P., P. van der Ley, K.E. Wilks, D.J. Maskell, J.T. Poolman, and E.R. Moxon. 1993. Mol Microbiol 10: 361-369), *lsi* (Jennings, M.P., M. Bisercic, K.L. Dunn, M. Virji, A. Martin, K.E. Wilks, J.C. Richards, and E.R. Moxon. 1995. Microb Pathog 19: 391-407), *lgtA, lgtB* (Jennings, M.P., D.W Hood, I.R. Peak, M. Virji, and E.R. Moxon. 1995. Mol Microbiol 18: 729-740) *rfaC* (Stoiljkovic, I., V. Hwa, J. Larson, L. Lin, M. So, and X. Nassif. 1997. FEMS Microbiol Lett 151: 41-49), *icsA* and *icsB* (van der Ley, P., M. Kramer, A. Martin, J.C. Richards, and J.T. Poolman, 1997. FEMS Microbiol Lett 146: 247-253) (Table 1). Other wild type *Neisseria meningitidis* strains used in the study were from three collections: 1) representatives of immunotypes L1-L12 (Poolman, J.T., C.T.P. Hopman, and H.C. Zanen. 1982. FEMS Microbiol Lett 13: 339-348); 2) global collection of 34 representative *Neisseria meningitidis* Group B strains (Seiler, A., R. Reinhardt, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19: 841-856); 3) global collection of 100 strains from 107 representative *Neisseria meningitidis* strains of all major serogroups (A, B, C, W, X,Y, Z) (Maiden, M.C.J., J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, I.M. Feavers, M. Achtman, and G.B. Spratt. 1998. PNAS 95: 3140-3145).

Capsule deficient and *galE* mutants were constructed in six *Neisseria meningitidis* Group B strains obtained from the collection as described in (Seiler, A., *et al.*, 1996. Mol Microbiol 19: 841-856) (Table 1). Other related *Neisseria* strains studied included 10 strains of *Neisseria gonorrhoeae* and commensal strains *lactamica* (8 strains), *polysaccharea* (1 strain),

*mucosa* (1 strain), *cinerea* (1 strain), *elongata* (1 strain), *sicca* (1 strain) and *subflava* (1 strain). Other Gram negative organisms included: *Haemophilus influenzae* type b (7 strains), *Haemophilus somnus* (1 strain), non-typable *Haemophilus influenzae* (8 strains), *Escherichia coli* (1 strain) and *Salmonella typhimurium* (1 strain) and its isogenic LPS mutants (*rfaC*, *rfaP*, *rfa1*) (Table 1).

**Bacterial culture in vitro**

All strains were grown overnight at 37°C on standard BHI medium base (Oxoid) in an atmosphere of 5% $CO_2$.

**Bacterial culture in vivo using the Chick Embryo Model**

To determine the accessibility of inner core epitopes of *Neisseria meningitidis* grown *in vivo* the chick embryo model was used (Buddingh, G.J., and A. Polk. 1937. Science 86: 20-21, Buddingh, G.J., and A. Polk. 1939. J Exp Med 70: 485-498, Schroten, H., M. Deadman, and E.R. Moxon. 1995. Pediar. Grenzgeb. 34: 319-324). The method was modified using an inoculum of $10^4$ and $10^5$ *Neisseria meningitidis* organisms in a final volume of 0.1ml, to infect the chorio-allantoic fluid of 10 day old Pure Sussex chick eggs (obtained from the Poultry Unit, Institute of Animal Health, Compton, Berks). After overnight incubation (37°C) the allantoic fluid (approx. 3-5mls) was removed from the eggs and the bacteria recovered after centrifugation at 350 x g for 15 minutes. The organisms were washed in sterile phosphate buffered saline (PBS) and stored in Greaves solution (5% BSA, 5% Sodium Glutamate, 10% Glycerol) at -70%°C.

**LPS extraction**

LPS samples were obtained from an overnight growth of *Neisseria meningitidis* plated on 5 BHI plates from which the organisms were scraped and suspended in 30 ml 0.05% phenol in PBS and incubated at room temperature for 30 minutes. Alternatively, batch cultures were prepared in fermenters using bacteria from an overnight growth (6 plates) in 50ml Difco Bacto Todd Hewitt broth (Difco) to inoculate 2.5L of the same medium. For insertion mutant strains, the medium contained 50µg/ml kanamycin. Following incubation at 37°C for 6-8h the culture was inoculated into 60L of Bacto Todd Hewitt broth in a New Brunswick

Scientific 1F-75 fermenter. After overnight growth (17h at 37°C), the culture was killed by addition of phenol (1%), and chilled to 15°C and the bacteria were harvested by centrifugation (13,000g for 20min) (Wakarchuk, W., *et al.*, 1996. J Biol. Chem. 271: 19166-19173). In either case, the crude LPS was extracted from the bacterial pellet using the standard hot phenol-water method (Westphal, O., and J. K. Jann. 1965. Meth. Carbohydr. Chem. 5:83-91) and purified from the aqueous phase by repeated ultracentrifugation (105,000 x g, 4°C, 2 x 5h) (Masoud, H., E.R. Moxon, A. Martin, D. Krajcarski, and J.C. Richards. 1997. Biochemistry 36: 2091-2103).

**Tricine gels**

Equivalent amounts of whole-cell lysates of *Neisseria meningitidis* strains or purified LPS were boiled in dissociation buffer and separated on standard tricine gels (30mA for 18h) (Lesse, A.J., A.A. Campagnari, W.E. Bittner, and M.A. Apicella. 1990. J Immunol Methods 126: 109-117). Gels were fixed and silver-stained as per manufacturers instructions (BioRad). To determine the presence of sialic acid, whole cell lysates were incubated with 2.5µl neuraminidase at 37°C for 18-20h (4U/ml Boehringer 1585886) and then with 5µl proteinase K at 60°C for 2-3h to remove proteins (Boehringer 1373196) prior to separation on tricine gels (16.5%).

**Characterization of LPS from MAb B5 negative strains**

LPS from wild-type and *galE*, *cap*- mutant MAb B5 negative strains were O-deacylated with anhydrous hydrazine as described previously (Masoud, H., E.R. Moxon, A. Martin, D. Krajcarski, and J.C. Richards. 1997. Biochemistry 36: 2091-2103). O-deacylated LPS were analysed by electrospray mass spectrometry (ES-MS) in the negative ion mode on a VG Quattro (Fisons Instruments) or API 300 (Perkin-Elmer/Sciex) triple quadruple mass spectrometer. Samples were dissolved in water which was diluted by 50% with acetonitrile : water : methanol : 1% ammonia (4:4:1:1) and the mixture was enhanced by direct infusion at 4µl/min. Deacylated and dephosphorylated LPS (L8 odA HF) was prepared according to the following procedure. LPS (160mg) was treated with anhydrous hydrazine (15ml) with stirring at 37°C for 30 minutes. The reaction was cooled (0°C), cold acetone (-70°C, 50ml) was added gradually to destroy excess hydrazine, and precipitated O-deacylated LPS (L8 odA)

was obtained by centrifugation. L8 odA was washed twice with cold acetone, and redissolved in water and lyophilised. The structure of L8 odA was confirmed by negative ion ES-MS before proceeding to dephosphorylation. L8 odA was dephosphorylated by treatment with 48% aqueous hydrogen fluoride (10ml) at 0°C for 48h. The product was dialysed against water, and the O-deacylated, dephosphorylated LPS sample (L8 odA HF) was lyophilised (50mg). Loss of phosphate was confirmed by ES-MS.

**Molecular modelling**

Molecular modelling of LPS epitopes was carried out as described previously by Brisson, J.R., S. Uhrinova, R.J. Woods, M. van der Zwan, H.C. Jarrell, L.C. Paoletti, D.L. Kasper, and H. Jennings. 1997. Biochemistry 36: 3278-3292). The starting geometry for all sugars was submitted to a complete refinement of bond lengths, valence and torsion angles by using the molecular mechanics program MM3(92) (QPCE). All calculations were performed using the minimised co-ordinates for the methyl glycoside. The phosphorus groups were generated from standard co-ordinates (Alchemy, Tripos software) and minimum energy conformations found in crystal structures. Calculations were performed using the Metropolis Monte Carlo (MMC) method. All pendant groups were treated as invariant except for the phosphorus groups which were allowed to rotate about the Cx-Ox and Ox-P bonds. The starting angles for the oligosaccharide were taken from the minimum energy conformers calculated for each disaccharide unit present in the molecule. 24-dimensional MMC calculations of the hexasaccharides with or without PEtn groups attached were carried out with 5000 macro moves. The graphics were generated using the Schakal software (Egbert Keller, Kristallographisches Institut der Universität, Freibury, Germany).

**Antibodies**

**Rabbit polyclonal antibody**

We used a rabbit polyclonal antibody specific for Group B *Neisseria meningitidis* capsular polysaccharide obtained by immunising a rabbit six times sub-cutaneously with lysates of MC58 at 2-week intervals. The first and second immunisations contained Freund's complete adjuvant and Freund's incomplete adjuvant respectively. Serum was obtained from bleed 6.

To increase specificity for the Group B capsular polysaccharide, rabbit polyclonal antibody (1 ml) was incubated overnight at 4°C with ethanol-fixed capsule-deficient MC58 ($5x10^9$ org./ml). This pre-adsorbed polyclonal antibody did not react with a capsule-deficient mutant of MC58 using immunofluorescence microscopy.

**Monoclonal antibodies to inner core LPS**

Murine monoclonal antibodies to H44/76 *galE* LPS were prepared by standard methods. Briefly, 6-8 week old Balb/c mice were immunised three times intraperitoneally followed by one intravenous injection with formalin-killed *galE* mutant whole cells. Hybridomas were prepared by fusion of spleen cells with SP2/O-Ag 14 (Shulman, M., C.D. Wilde, and G. Kohler. 1978. Nature 276: 269-270) as described (Carlin, N.I., M.A. Gidney. A.A. Lindberg, and D.R. Bundle. 1986. J Immunol 137: 2361-2366). Putative hybridomas secreting *galE* specific antibodies were selected by ELISA employing purified LPS from L3 and its *galE* mutant and L2. Ig class, subclass and light chain were determined by using an isotyping kit (Amersham Canada Ltd, Oakville, Ontario). Clones were expanded in Balb/c mice following treatment with pristane to generate ascitic fluid. Spent culture supernatant was collected following in vitro culture of hybridoma cell lines. Further testing of *galE* MAbs was carried out by screening against purified LPS from *Neisseria meningitidis* L3 *lgtA*, *lgtB* and *lgtE* mutant strains (Figure 1), and *Salmonella typhimurium* Ra and Re mutants. One of the MAbs, MAb B5 ($IgG_3$), was selected for more detailed study.

**Immunotyping monoclonal antibodies**

To determine the immunotypes of *Neisseria meningitidis* Strains studies, especially L2 and L4-L6, the following murine MAbs were used in dot blots and whole cell ELISA: MN42F12.32 (L2,5), MN4A8B2 (L3,7,9), MN4C1B (L4,6,9), MN40G11.7 (L6), MN3A8C (L5) (Scholten, R.J., *et al.*,. J Med Microbiol 41: 236-243).

**Human umbilical vein endothelial cell (HUVEC) assay**

Cultured human umbilical vein endothelial cells (HUVECs) were prepared as described previously (Virji, M., *et al.*, 1991. Microb Pathog 10: 231-245) and were infected with strains of *Neisseria meningitidis* for 3h at 37°C. *Neisseria meningitidis* strains were grown wither *in*

*vitro* or *in vivo* using the chick embryo model (as described above). The accessibility of the inner core LPS epitopes of whole-cell *Neisseria meningitidis* to specific MAb B5 was determined using immunofluorescence and confocal microscopy. Gelatin-coated glass coverslips coated with HUVECs were infected with wild-type *Neisseria meningitidis* as described previously (Virji, M., *et al.*, 1991. Mol Microbiol 5: 1831-1841), except bacteria were fixed with 0.5% paraformaldehyde for 20 min instead of methanol. For accessibility studies, coverslips were washed with PBS, blocked in 3% BSA-PBS and incubated with MAb B5 culture supernatant and pre-adsorbed polyclonal rabbit anti-capsular antibody. Binding of antibody to wild-type *Neisseria meningitidis* strains was detected by anti-mouse IgG rhodamine (TRITC) (Dako) and anti-rabbit IgG fluorescein (FITC) (Sigma). HUVECs were stained using diaminophenylamine DAPI (1μg/ml) (Sigma). Mounted coverslips were viewed for immunofluorescence using appropriate filters (Zeiss Microscope with Fluorograbber, Adobe Photoshop or confocal microscope (Nikon Model).

**ELISA**

**Purified LPS ELISA**

A solid phase indirect ELISA employing purified LPS was used to determine the binding specificities of MAbs. Nunc maxisorp plates were coated overnight with 1.0μg/well of purified LPS derived from wild type and mutants. LPS (10μg/ml) was diluted in 0.05M carbonate buffer containing 0.02M $MgCl_2$, pH 9.8. Non-specific binding sites were blocked for 1h with 1% BSA-PBS (Sigma) and washed three times with PBS Tween 20 (0.05% v/v) (PBS-T). Plates were incubated for 1h with MAb B5 culture supernatant and washed three times in PBS-T. Primary antibody was detected using anti-mouse IgG-alkaline phosphatase (Sigma: Cedarlane Laboratories Ltd.) incubated for 1h, washed three times in PBS-T, and detected using p-nitrophenyl phosphate AP substrate system (Sigma: Kirkegaard & Perry Laboratories). The reaction was stopped after 1h with 50μl 3M NaOH and absorbance determined at OD $A_{405-410nm}$ (Dynatech EIA plate reader).

**Inhibition ELISA**

For inhibition ELISA studies, MAb B5 was incubated with purified LPS samples prior to addition to L3 *galE* LPS coated plates and assayed as described above.

**Whole cell ELISA**

Whole cell (WC) ELISA was performed using heat-inactivated lysates of *Neisseria meningitidis* organisms as described previously (Abdillahi, H., and J.T. Poolman. 1988. J Med Microbiol 26:177-180). Nunc Maxisorp 96-well plates were coated with 100µl bacterial suspension (OD of 0.1 at $A_{820nm}$) overnight at 37°C, blocked with 1% BSA-PBS and identical protocol followed as for LPS ELISA.

**Dot blots**

Bacterial suspensions prepared as above (2µl) were applied to a nitrocellulose filter (45 micron, Schleicher and Schueller) and allowed to air dry. The same procedure as described for WC ELISA was followed except the detection substrate was 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue-tetrazolium (BCIP/NBT) (2mg/ml; Sigma). The colour reaction was stopped after 30 min by several washes with PBS and blots were air-dried.

**Results**

To investigate the potential of inner core LPS structures of *Neisseria meningitidis* as vaccines, we have studied the reactivity of an isotype $IgG_3$ murine monoclonal antibody (MAb), designated B5, raised against *Neisseria meningitidis* stain H44/76 immunotype L3 *galE* mutant. MAb B5 was one of seven monoclonal antibodies to LPS inner core produced against *Neisseria meningitidis* immunotype L3 *galE* by standard immunological methods (see Methods). Preliminary ELISA testing showed B5 cross-reacted with LPS from L3 parent strain and with *galE* (*lgtE*), *lgtA* and *lgtB* mutants, but did not cross-react with *Salmonella typhimurium* Ra or Re LPS.

In order to determine the specific inner core epitope recognised by MAb B5, various *Neisseria meningitidis* strains of known structure were examined in ELISA for cross reactivity (Figure 2). The most significant finding of this analysis was that *Neisseria meningitidis* immunotype L4 LPS was not recognised by MAb B5. The only structural difference between

immunotypes L4 and L3 (which is recognised by MAb B5) is the position of attachment of the PEtn group (Figure 3). In immunotype L3 LPS the PEtn is attached at the 3-position of HepII, whereas in immunotype L4 LPS the PEtn is attached at the 6- or 7- position of HepII (Figure 3). Additionally, LPS from immunotype L2 and its *galE* mutant (in which the PEtn group is attached at the 6-position and a glucose residue is present at the 3-position of HepII) are not recognised by MAb B5. Immunotype L5, which has no PEtn in the inner core, is not recognised by B5, whereas immunotype L8 and its *galE* mutant which have PEtn at the 3-position of HepII are recognised. These results suggest that MAb B5 specifically recognises PEtn when it is attached at the 3 -position of HepII.

In order to prove the essential inclusion of PEtn in the epitope recognised by MAb B5, immunotype L8 O-deacylated (odA) LPS was dephosphorylated (48% HF, 4°C 48h) (Figure 3). The absence of PEtn following dephosphorylation was confirmed by ES-MS analysis. As indicated in Figure 4, dephosphorylation of L8 odA LPS abolished reactivity to MAb B5. To further characterise the epitope recognised by MAb B5, several structurally defined genetic mutants of immunotype L3 were screened for cross-reactivity (Figure 4). The highly truncated LPS of mutant strain *icsB* was only weakly recognised, while mutant strain *icsA* LPS was not recognised by MAb B5. These results suggest that the presence of glucose on the proximal heptose reside (HepI) is not absolutely necessary for binding by B5 but is required for optimal recognition (Figure 1). Furthermore, MAb B5 does not bind LPS in which both the glucose on the α-chain, HepI, and the N-acetylglucosamine residue on the β-chain, HepII, are absent. This suggests that the presence of N-acetylglucosamine is required to present the PEtn residue in the correct conformation for binding by MAb B5. Genetic modifications that produce severely truncated LPS glycoforms were also examined for reactivity with MAb B5. LPS from immunotype L3 *lsi* which has a trisaccharide of Hep-Kdo-Kdo attached to lipid A, and L3 PB4 which only contains the Kdo disaccharide and lipid A were not recognised by MAb B5 (Figure 4). Inhibition ELISA studies (data not shown) were in accord with this result, thus confirming the specificity of MAb B5 to the PEtn molecule linked at the 3-position of HepII.

To demonstrate the ability of MAb B5 to recognise this inner core epitope in encapsulated strains, we devised an assay in which natural isolates of *Neisseria meningitidis* were studied when they were grown on and became adherent to tissue cultured cells (HUVECs). Initially, this methodology was developed using the fully encapsulated strain MC58. The advantages of using the HUVEC assay were that they provided a monolayer of endothelial cells to which the bacteria could adhere and that they provided a biologically relevant environment. Previous attempts using *Neisseria meningitidis* directly adherent to gelatin- or matrigel-coated coverslips resulted in low numbers of adherent bacteria after repeated washings and high non-specific background staining.

Primary antibodies, MAb B5 and a polyclonal anti-capsular antibody were detected by anti-mouse TRITC and anti-rabbit FITC respectively. This demonstrated that an inner core LPS epitope of the fully encapsulated strain (MC58) was accessible to MAb B5 (Figure 5a). Confocal microscopy showed that MAb B5 and anti-capsular antibodies co-localised. In addition to this *in vitro* demonstration of accessibility of MAb B5 to inner core LPS, we also investigated organisms grown *in vivo* using the chick embryo model. Strain MC58 ($10^4$ org./ml) was inoculated into chorio-allantoic fluid of 10 day old chick embryos and harvested the next day to provide *ex-vivo* organisms. The results of confocal microscopy were identical to those observed *in vitro*, that is MAb B5 and anti-capsular antibodies co-localised (Figure 5b). This demonstrated that the inner core LPS epitopes were also accessible *in vivo* on whole-encapsulated wild-type *Neisseria meningitidis*.

The observation of double staining of the inner core LPS epitope in the presence of capsule is key to the concept of this approach and therefore a number of controls were used to confirm the validity finding. These included: (i) double staining a MAb B5 negative e.g. immunotype L4 strain with MAb B5 and anti-capsular antibody. This resulted in no reactivity of MAb B5 on rhodamine filter but positive reactivity with anti-capsular antibody. This rules out a band-passing effect during the recording of the pictures; (ii) single staining of encapsulated MAb B5 positive strains with either MAb B5 alone or anti-capsular antibody alone followed by staining with rhodamine or FITC, respectively. When viewed on the appropriate wavelength there was no cross-reactivity during immunofluorescent staining nor any band-passing effect;

(iii) double-staining of a MAb B5 positive or negative strain without capsule with MAb B5 and anti-capsular antibody resulted in no capsular staining but either MAb B5 positive or negative reactivity when viewed on the rhodamine filter. This excluded cross-reactivity during staining or band-passing effect resulting in artefactual inner core staining.

To survey the extent of MAb B5 reactivity with other *Neisseria meningitidis* strains, three collections were investigated.

i) 12 strains representative of LPS immunotypes L1-L12

ii) 34 Group B strains selected to represent genetically diverse isolates from many different countries obtained between the years 1940-1988 (Seiler, A., R. Reinhardt, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19: 841-856)

iii) a global collection of 107 genetically diverse strains representing all capsular serogroups, also obtained from different countries from 1940-1994 (Maiden, M.C.J., J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, I.M. Feavers, M. Achtman, and G.B. Spratt. 1998. PNAS 95: 3140-3145).

Of the 12 immunotypes, MAb B5 recognised the LPS of strains in which the inner core oligosaccharide has a PEtn linked to the 3-position of HepII (Table 2 and Figure 1). Thus, immunotypes L2, L4, L6 did not react with MAb B5, whereas immunotypes L1, L3, L7-L12 were recognised by MAb B5. This confirmed that the presence of PEtn in the 3 position of the HepII is necessary to confer MAb B5 reactivity (Figure 3).

To investigate further the MAb B5 reactivity with other Group B strains, a collection of genetically diverse strains was studied (Seiler, A., R. Reinhardt, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19: 841-856). MAb B5 reactivity was detected in 26/34 (76%) of Group B *Neisseria meningitidis* stains tested. This included representative strains of ET-5, ET-37, A4 and Lineage-3. This represents the most complete available collection of hyper-invasive lineages of *Neisseria meningitidis* Group B strains.

We obtained capsule-deficient and *galE* mutants from six of eight of the MAb B5 negative Group B strains (transformations were unsuccessful in the other two strains). Theses were

also negative with MAb B5 using dot blot, whole cell ELISA or immunofluorescence, with the exception of a BZ157 *galE cap-* mutant; which had low level reactivity both by immunofluorescence and dot blot. The MAb B5 strains were characterised using a battery of immunotyping MAbs. We determined the immunotype of the eight MAb B5 negative strains using combinations of the appropriate MAbs (see Methods) and dot blots of WC lysates (obtained from Peter van der Ley) (Table 3). In addition, structural fingerprinting of the inner core region of MAb B5 negative strains was performed by ES-MS on O-deacylated LPS from five of the respective capsule-deficient *galE* mutants (1000, NGE30, EG327, BZ157, NGH38) (Table 4). Strains 1000, NGE30, EG327 were non-typical by MAbs and LPS from these strains lacked PEtn on HepII of the inner core. BZ157, which corresponded to immunotype L2 by MAbs contained PEtn in the inner core, and by analogy to L2 at the 6/7 position of HepII (Table 3). NGH38 was immunotype L2, L5 and analogous to L2 by structural analysis. Those strains that were non-typable failed to react with MAbs that recognise L3,7,9, L6, L2,5, L4,6,9. However, 15/17 MAb B5 negative *Neisseria meningitidis* strains (all serogroups) were positive for L2, 5 and all MAb B5 positive strains were positive for L3,7,9,. No reaction with any immunotyping MAbs was observed with 8/32 MAb B5 negative strains and 24/68 of MAb B5 positive strains.

To determine if the degree of sialylation of the LPS was a factor in the ability of MAb B5 to recognise its inner-core epitope, MAb B5 negative strains were examined by LPS gels. MAb B5 reactivity was unaffected by varying the state of sialylation through exposure to neuraminidase as described in methods (Figure 6). Furthermore, strain MC58, with which the MAb B5 reacted strongly, was found to be highly sialylated (Figure 6) and this was confirmed by ES-MS of purified O-deacylated LPS (data not shown). Therefore our data did not support a contribution of sialylation to the lack of MAb B5 reactivity.

With respect to the other *Neisseria* species, MAb B5 also recognised the inner core LPS of five strains of *Neisseria gonorrhoeae* (F62, MS11, FA19, 179008, 150002) (two were negative) and (at least) two strains of *Neisseria lactamica* (L19, L22). However, MAb B5 did not react with one strain each of *Neisseria polysaccharea* (M7), *Neisseria mucosa* (F1), *Neisseria cinerea* (Griffiss, J.M., J.P. O'Brien, R. Yamensaki, G.D. Williams, P.A. Rice, and

H. Schneider. 1987. Infect Immun 55: 1792-1800), *Neisseria elongata* (Q29), *Neisseria sicca* (Q39) and *Neisseria subflava* (U37). Also MAb B5 did not react with *Escherichia coli* (DH5 alpha), *Salmonella typhimurium* (LT2) or its isogenic LPS mutants (*rfaC*, *rfaI*, *rfaP*).

Finally, we investigated the reactivity of MAb B5 with 100 strains that included representatives of serogroups A, B, C, W, X, Y and Z (Maiden, M.C.J., *et al.*, 1998. PNAS 95: 3140-3145). Of these strains, 70% were MAb B5 positive. Clustering according to genetic relatedness was evident. For example, none of the MAb B5 negative stains were in the ET5 complex. Among Group A strains, MAb B5 positive and negative stains also fell into distinct clusters. For example, lineages I-III and lineage A4 were positive and lineage IV-I was negative. This collection, together with that described in (Seiler, A., *et al.*, 1996. Mol Microbiol 19: 841-856) represents the most complete set available for known hyper invasive lineages in all major serogroups of *Neisseria meningitidis* strains.

**Discussion and Conclusions**

The pre-requisites for any candidate *Neisseria meningitidis* Group B vaccine would be that in contains a highly conserved epitope(s) that is found in all Group B stains and is accessible to antibodies in the presence of capsule. Our approach has combined genetics, structural analysis and immunobiology to define candidate epitopes in inner core LPS of *Neisseria meningitidis* Group B. This study uses murine MAb B5, isotype IgG3, which was raised to a genetically defined immunotype L3 *galE* mutant in order to specifically target inner-core LPS epitopes. The epitope(s) recognised by MAb B5 was defined by cross-reactivity studies with purified LPS glycoforms of known structure. MAb B5 recognised all LPS glycoforms in which the PEtn is at the 3-position of HepII (immunotypes L1, L3, L7, L8 and L9) and failed to react with immunotypes where PEtn is at the 6- or 7- position (L2, L4 and L6) or absent from HepII (L5) (Figure 1). MAb B5 reacted with 70% *Neisseria meningitidis* strains tested from the two most complete sets of *Neisseria meningitidis* strains available word-wide (Seiler, A., *et al.*, 1996. Mol Microbiol 19: 841-856, 35). Of these strains, 76% of *Neisseria meningitidis* Group B strains tested were positive with MAb B5 and 70% of a collection that included all *Neisseria meningitidis* serogroups tested was positive with MAb B5. Therefore,

it may be envisaged that a vaccine containing a limited number of glycoforms, representing all the possible PEtn positions (none, 3 and 6/7) on HepII on the inner core, would cover 100% of *Neisseria meningitidis* Group B strains.

The LPS structures of MAb B5 negative strains were confirmed by structural analysis. Two structural variants were recognised. One variant without PEtn in the inner core LPS (e.g. NGE30, EG327, 1000); and the other, with PEtn group of HepII (e.g. BZ157, NGH38) at the 6- or 7- position instead of the 3-position.

With a view to developing inner core LPS epitopes as vaccine candidates, it is significant that there were no effects of the capsule on MAb B5 accessibility, as shown by co-localisation of the anti-capsule antibody and MAb B5 in wild-type organisms (MC58) grown *in vitro* and *in vivo* by confocal microscopy (Figures 5a and b). Nor did the presence or absence of sialic acid have an effect since both MAb B5 positive and negative strains had high sialylation states as shown by tricine gels (Figure 6) and confirmed by ES-MS (data not shown).

There was no evidence of phase variation in MAb B5 positive or negative strains in this study, with the exception of one strain (BZ157) which had a very low level of MAb B5 positive strains in parent and *galE* mutant (0.06%) (data not shown). Structural analysis of LPS extracted from these two variants is currently under investigation.

Three dimensional space filling models of the inner core LPS of L3 and L4 immunotypes show that the position of the PEtn, either 3- or 6-position respectively (shown in brown), alters the accessibility and conformation of PEtn in the inner core epitope (Figure 3). The most striking example of the importance of PEtn for MAb B5 reactivity was observed when PEtn was removed from the immunotype L8 (MAb B5 positive) by treatment with hydrogen fluoride (HF) which totally abolished MAb B5 reactivity.

Previous studies with oligosaccharide conjugates in mice and rabbits have demonstrated that PEtn is important in immunogenicity and functional activity of polyclonal antibodies (Verheul, A.F., *et al.*, 1991. Infect Immun 59: 843-851). These studies identified two sets of

polyclonal antibodies. One set resulting from L1 and L3,7,9 oligosaccharides had PEtn in the 3-position of HepII, were immunogenic, had opsonophagocytic (OP) and chemiluminescence in oxidative burst reaction, but had no serum bactericidal activity. The other set of antibodies resulting from L2 conjugates (6- or 7-position or without PEtn at HepII) were poorly immunogenic and had greatly reduced OP activity and chemiluminescence (Verheul, A.F., A.K. Braat, J.M. Leenhouts, P. Hoogerhout, J.T. Poolman, H. Snippe, and J. Verhoef. 1991. Infect Immun 59: 843-851). Future studies will look at the safety and immunogenicity of inner core LPS-conjugates (PEtn at 3-position of HepII and alternative glycoforms) and the functional ability of these polyclonal antibodies in opsonic and serum bacterial assays, initially in mice and rabbits. Preliminary studies using MAb B5 in an opsonophagocytosis assays with *Neisseria meningitidis* strain MC58 and donor human polymorphonuclear cells suggest MAb B5 is opsonic in the presence of complement and that the uptake of *Neisseria meningitidis* bacteria correlates with an oxidative burst reaction within the neutrophil. MAb B5 does not appear to have any significant serum bactericidal activity with *Neisseria meningitidis* strain MC58, however this is not unexpected in view of its isotype (IgG3). The functionality of MAb B5 is currently under further investigation.

In conclusion, MAb B5 recognises a conserved inner core epitope in which the PEtn is at the 3-position of HepII. This epitope was present in 76% *Neisseria meningitidis* Group B strains and 70% of all *Neisseria meningitidis* serogroups, and was accessible in the presence of capsule. A limited number of alternative glycoforms have been identified that are not recognised by MAb B5 where the PEtn is either absent or at an exocyclic position of HepII. Therefore, a vaccine containing a limited number of glycoforms might give 100% coverage of all *Neisseria meningitidis* Group B strains.

**Table 1. Bacterial strains.**

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| *Neisseria meningitidis* | | |
| MC58 | **L3** | CSF isolate<br>Virji, M., H. Kayhyt, D.J.P. Ferguson, J.E. Heckels, and E.R. Moxon. 1991. Mol Microbiol 5:1831-1841. |
| H44/76 | **L3** | Holton, E. 1979. J Clin Microbiol 9:186-188. |
| MC58 | *galE* | Jennings, M.P., P. van der Ley, K.E. Wilks, D.J. Maskell, J.T. Poolman, and E.R. Moxon. 1993. Mol Microbiol 10:361-369. |
| MC58 | *lsi1(rfaF)* | Jennings, M.P., M. Bisercic, K.L. Dunn, M. Virji, A. Martin, K.E. Wilks, J.C. Richards, and E.R. Moxon. 1995. Microb Pathog 19:391-407. |
| MC58 | *lgtA* | Jennings, M.P., D.W. Hood, I.R. Peak, M. Virji, and E.R. Moxon. 1995. Mol Microbiol 18:729-740. |
| MC58 | *lgtB* | Jennings, M.P., D.W. Hood, I.R. Peak, M. Virji, and E.R. Moxon. |

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| | | 1995. Mol Microbiol 18:729-740. |
| H44/76 | *rfaC* | Stolljokovic, I., V. Hwa, J. Larson, L. Lin, M. So, and X. Nassif. 1997. FEMS Microbial Lett 151:41-49. |
| H44/76 | *icsA* | van der Ley, P., M. Kramer, A. Martin, J.C. Richards, and J.T. Poolman. 1997. FEMS Microbiol Lett 146:247-253. |
| H44/76 | *icsB* | van der Ley, P., M. Kramer, A. Martin, J.C. Richards, and J.T. Poolman. 1997. FEMS Microbiol Lett 146:247-253. |
| 126E; 35E;H44/76;89I;M981; M992 6155;892257;M978;120 M;7880; 7889;3200 | **L1-L12 RESPECTIVELY** | Poolman, J.T., C.T.P. Hopman, and H.C. Zanen. 1982. FEMS Microbial Lett 13:339-348. |
| BZ157 | **L2** | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| BZ157 | *galE* | This study |
| 1000 | **NT** | Seiler, A., R. Reinhart, J. Sakari, |

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| | | D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| 1000 | *galE* | This study |
| NGE30 | **NT** | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| NGE30 | *galE* | This study |
| EG327 | **NT** | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| EG327 | *galE* | This study |
| NGH38 | **L2,5** | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| NGH38 | *galE* | This study |
| EG328 | **NT** | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |
| EG328 | *galE* | This study |
| 3906;NGH15;BZ133;BZ83;EG329; SWZ107;BZ198;NGH4 | | Seiler, A., R. Reinhart, J. Sakari, D.A. Caugant, and M. Achtman. 1996. Mol Microbiol 19:841-856. |

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| 1;NG4/88; 2970;BZ147;NGG40;N GH36;NG3/88; NGF26;NG6/88;NG H38;NGE28;BZ169; 528;DK353;BZ232;DK 24;BZ159;BZ10; BZ163;NGP20 B40;Z4024;Z4081;Z24 91;Z3524;Z3906;Z5826 ;BZ10;BZ163;B6116/7 7;L93/4286; NG3/88;NG6/88;NGF2 6;NGE31;DK24; 3906;EG328;EG327;10 00;B534;A22;71/94;86 0060;NGG40;NGE28; NGH41;890326;860800 ;NG4/88;E32;44/76;204 /92;BZ8; SWZ107; NGH38;DK353; BZ232; E26; 400; BZ198; 91/40; NGH15; NGE30; 50/94; 88/03415; NGH36; BZ147;297-0 | | (35) |
| *Neisseria lactamica* | | Brian Spratt & Noel Smith |

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| *(L12,L13,L17,L18,L19, L20,L22) polysaccharea (P4),mucosa (M7),cinerea (F1),elongata (I8), sicca (Q29). subflava (U37)* | | |
| *Neisseria gonorrhoeae:* | | |
| F62,MS11,FA19,FA10 | | R.Goldstein |
| 90,179008, 150002,15253 | | |
| SN-4 | | Staffan Normavk |
| P9-2 | | M.Virji |
| *Haemophilus influenzae* type b | | |
| Eagan; 7004; Rd | *opsx* | Hood, D.W., M.E. Deadman, T. Allen, H. Masoud, A. Martin, J.R. Brisson, R. Fleischman, J.C. Venter, J.C. Richards, and E.R. Moxon. 1996. Mol Microbiol 22:951-964. |
| 5B33; 3Fe;E3Fi;E1B1 | *rfaF* | |
| | *orfH* | |
| | *lpxA* | |
| PLAK33 | | Steeghs, L., R. den Hartog, A. den Boer, B. Zomer, P. Roholl, and P. van der Ley. 1998. Nature 392:449-450. |
| *Haemophilus somnus*; | | J.Richards |

| Species Strain | Relevant immunotype (bold) and genotype (italics) | Source/reference |
|---|---|---|
| 738 L1 Non-typable *Haemophilus influenzae* (NTHI): 54, 375, 477, 1003, 1008, 1042, 1147,1231 | | J. Eskola |
| *E. coli* DH5a | | Neidardt, F.C. 1996. Roy Curtiss III, J.L. Ingraham, E.C. Lin, K. Brooks, B. Magasanik, W.S. Reznikoff, M. Riley, S.M. and H.E. Umbarger (ed.), ASM Press. |
| *Salmonella typhimurium* LT2 | *rfaC* *rfaI* *rfaP* | Schnaitman, C.A., and F.D. Klena. 1993. 57:655-682. |

Table 2. Reactivity of monoclonal antibody B5 with representative *Neisseria meningitidis* strains of immunotypes LI-L12 determined by whole cell ELISA, dot blots of lysates, immunofluorescence and confocal microscopy.

| Strain | Serogroup: Serotype: Serosubtype | Immuno-type | Whole cell ELISA [a] ($OD_{A405nm}$) | Dot Blot [b] | Immuno-fluorescence [c] |
|---|---|---|---|---|---|
| 126E | C:3:P1.5,2 | L1 | + 1.8 | +++ | + |

| | | | | | |
|---|---|---|---|---|---|
| 35E | C:20:P1.1 | L2 | - <0.4 | - | - |
| H44/76 | B.15.P1.7,16 | L3 | + 1.3 | +++ | ++ |
| 89I | C:nt:P1.16 | L4 | -<0.4 | - | - |
| M98I | B:4:P1.- | L5 | - <0.4 | +/- | - |
| M992 | B:5:P1.7,1 | L6 | - <0.4 | +/- | - |
| 6155 | B:nt:P1.7,1 | L7 | + 0.8 | ++ | + |
| M978 | B:8:P1.7,1 | L8 | + 1.9 | +++ | ++ |
| 892257 | B:4:P1.4 | L8 | + 1.9 | | |
| 120M | A:4:P1.10 | L9 | + 1.8 | +++ | + |
| 7880 | A:4:P1.6 | L10 | + 2.2 | +++ | + |
| 7889 | A:4:P1.9 | L11 | + 2.0 | +++ | ++ |
| 3200 | A:4:P1.9 | L12 | + 2.1 | +++ | ++ |

[a] Positive reactivity ($OD_{A405} > 0.4$) (+), negative reactivity ($OD_{A405} < 0.4$) (-).

[b] Strongly positive (+++), positive (++), weakly positive (+/-), negative (-).

[c] Strongly positive (++), positive (+), negative (-).

**Table 3**

Correlation between reactivity with monoclonal antibody B5, immunotyping and location of phosphoethanolamine (PEtn) on HepII of inner core.

| Strain | MAb B5 | Immuno-type | Position of PEtn on HepII | |
|---|---|---|---|---|
| | | | O-3 | O-6 |
| MC58 | + | L3,7 | + | - |
| 1000 | - | NT | - | - |
| NGE30 | - | NT | - | - |
| EG327 | - | NT | - | - |
| BZ157 [‡] | - | L2,5 | - | + |
| BZ157 [§] | + | L3,7 | + | - |
| NGH38 | - | L2,5 | - | + |

Abbreviations:

NT= non-typable

*MN4A8B2 (L3,7,9);MN42F12.32 (L2,5);MN4C1B (L4,6,9);MN40G11.7 (L6).

‡BZ157 MAb B5 negative variant

§BZ157 MAb B5 positive variant

Table 4

Negative ion ES-MS data and proposed compositions of *O*-deacylated LPS from *galE* capsule-deficient mutant *Neisseria meningitidis* MAb B5 negative strains. Average mass units were used for calculation of molecular weight based on proposed composition as follows: Glc, 162.15; Hep, 192.17; GlcNAc, 203.19; Kdo, 220.18; PEtn, 123.05.

| Strain | Observed Ions (*m/z*) | | Molecular Mass (Da) | | |
|---|---|---|---|---|---|
| | $(M-2H)^{2-}$ | $(M-H)^{-}$ | Observed | Calculated | Lipid A[b] |
| 1000 | 1213.0 | 2427.6 | 2427.7 | 2427.2 | 1075 |
| | 1252.9 | 2507.8 | 2507.8 | 2507.2 | 1155 |
| | 1314.5 | 2630.9 | 2630.9 | 2630.3 | 1278 |
| NGH38 | 1293.8 | 2589.5 | 2589.3 | 2589.3 | 952 |
| EG327 | 1151.2 | 2304.4 | 2304.4 | 2304.1 | 952 |
| NGE30 | 1132.1 | - | - | 2265.1 | 1075 |

| Strain | Observed Ions (*m/z*) $(M-2H)^{2-}$ | Observed Ions (*m/z*) $(M-H)^{-}$ | Molecular Mass (Da) Observed | Molecular Mass (Da) Calculated | Lipid A[b] |
|---|---|---|---|---|---|
| | 1396.1 | 2793.4 | 2793.7 | 2792.5 | 1075 |
| | 1436.0 | 2873.7 | 2873.9 | 2872.5 | 1155 |
| | 1498.0 | 2997.2 | 2997.1 | 2995.6 | 1278 |
| BZ157 | 1274.6 | 2551.4 | - | 2550.3 | 1075 |
| | 1314.8 | 2631.1 | 2631.2 | 2630.3 | 1155 |
| | 1376.4 | 2754.4 | 2754.5 | 2753.4 | 1278 |
| | 1457.5 | 2916.6 | 2916.6 | 2915.6 | 1278 |

[a] Glc, glucose; GlcNAc, N-acetylglucosamine; PEtn, phosphoethanolamine; Hep, heptose; Kdo, 3-deoxy-D-manno-octulosonic acid.

[b] As determined by MS-MS analyses.

Table 4 continued

| Strain | Proposed Composition [a] |
|---|---|
| 1000 | 2Glc, GlcNAc, 2Hep, 2 Kdo, Lipid A |
| | 2Glc, GlcNAc, 2Hep, 2 Kdo, Lipid A |
| | 2Glc, GlcNAc, 2Hep, 2 Kdo, Lipid A |

| Strain | Proposed Composition [a] |
|---|---|
| NGH38 | 3Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A |
| EG327 | 2Glc, GlcNAc, 2Hep, 2 Kdo, Lipid A |
| NGE30 | Glc, GlcNAc, 2Hep, 2 Kdo, Lipid A |
| | 3Glc, 2GlcNAc, 2Hep, 2 Kdo, Lipid A |
| | 3Glc, 2GlcNAc, 2Hep, 2 Kdo, Lipid A |
| | 3Glc, 2GlcNAc, 2Hep, 2 Kdo, Lipid A |
| BZ157 | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A |
| | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A |
| | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A |
| | 3Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A |

[a] Glc, glucose; GlcNAc, N-acetylglucosamine; PEtn, phosphoethanolamine; Hep, heptose; Kdo, 3-deoxy-D-manno-octulosonic acid.

[b] As determined by MS-MS analyses.

**Figure Legends - Example 1**

Figure 1

Representation of the structure of meningococcal LPS oligosaccharides of immunotypes L1-L9. Immunotypes are indicated to the extreme left. The vertical dotted line marks the junction between the inner core structures to the right and outer core structures to the left. The epitope recognised by MAb B5 is indicated in bold (MAb B5 positive). Arabic numerals indicate the linkage between sugars or amino-sugars. Alpha and beta indicate the carbon 1 linkage at the non-reducing end of the sugar. Genes for incorporating each of the key sugars or amino-sugars into the LPS oligosaccharide in the biosynthetic pathway are indicated with arrows indicating where in the pathway the gene product is required. Abbreviations include: Kdo, 2-keto-2-deoxyoctulosonic acid; PEtn, phosphoethanolamine; Gal, galactose: GLcNAc, N-acetyl glucosamine; Glc, glucose; Hep, Heptose. Immunotype L5 has no PEtn on the second heptose. The gene that adds the glucose to the second heptose (*lgtG*) is phase-variable.

Figure 2

Cross-reactivity of MAb B5 with selected immunotypes and mutants of *Neisseria meningitidis* LPS and *O*-deacylated (odA) LPS as determined by solid phase ELISA. LPS glycoforms of immunotypes L2 (35E) (solid black bars), L3 (H44/76) (open bars), L4 (891) (diagonal line filled bars), L5 (M981), L8 (M978) (horizontal line filled bars), wild-type and respective mutants (*galE*, *lgtA* or *lgtB*), in a native or *O*-deacylated form, were coated onto ELISA plates (see methods) and reactivity of MAb B5 determined by standard ELISA (OD $A_{410nm}$).

Figure 3

Space-filling 3-D molecular models of the calculated (MMC) lowest energy states of the core oligosaccharide from *galE* mutants of (a) L3, (b) L4 and (c) L8-dephosphorylated. Kdo moiety indicated in grey is substituted at the O-5 position by the heptose disaccharide inner-core unit (red), HepI provides the point via a glucose residue (dark green) for extension to give α-chain epitopes, while HepII is substituted by N-acetyl glucosamine residue (lighter green) at O-2. PEtn (brown) is shown in O-3 position in L3 immunotype and O-6 in L4 immunotype. Colour versions of this and the other figures for Example 1 are to be found in Plested *et al.*, 1999 Infect. Immunity 67, 5417 - 5426.

Figure 4

Cross-reactivity of MAb B5 with genetically modified L3 LPS and chemically modified L8 LPS from *Neisseria meningitidis* as determined by solid phase ELISA. LPS glycoforms of immunotype L8 (M978) (horizontal line filled bars) chemically modified by O-deacylation and HF treatment and immunotype L3 (H44/76) (open bars) *galE, icsB, icsA, lsi,* PB4 mutants (*O*-deacylated) were coated onto ELISA plates (see methods) and reactivity of MAb B5 determined by standard ELISA ($OD_{A410nm}$).

Figure 5.a.

Confocal immunofluorescence microscopy of *Neisseria meningitidis* organisms, strain MC58 adherent to human umbilical vein endothelial cells (HUVECs). (a) Fluorescein tagging with rabbit polyclonal antibody specific for Group B *Neisseria meningitidis* capsule. (b) rhodamine tagging of MAb B5, specific for *galE* LPS (x2400 magnification). Confocal immunofluorescence microscopy of *in vivo* grown MC58 organisms stained as described in Plested *et al.*, 1999 Infect. Immunity 67, 5417-5426. (c) anti-capsular antibody (green). (d) MAb B5 (red) (x2400 magnification).

Figure 6

Silver-stained tricine gels of LPS preparations (10μg/lane) from *Neisseria meningitidis* Group B strains which were not reactive with MAb B5. These LPS preparations were either not treated (-) or treated with (+) neuraminidase to show the presence of sialic acid: a) MAb B5 negative strains Lanes 1,2 = NGE30; lanes 3,4 = BZ157; lanes 5,6 = EG328; lanes 7,8 = 1000; lanes 9,10 = 3906. b) MAb B5 negative strains: Lanes 1,2 = EG327; lanes 3,4 = NGH38; lanes 5,6 = NGH15; MAb B5 positive strain: lanes 7,8 = MC58. Presence of sialic acid (NeuAc) indicated by →. This band was seen in untreated (-) and removed in treated (+) neuraminidase preparations.

**Example 2 Identification of additional inner core epitopes**

## INTRODUCTION

Example 1 identifies an inner core LPS epitope that was accessible and conserved in 70% of a global collection of 104 *Neisseria meningitidis* strains representative of all major serogroups (Plested *et al.*, 1999, Infect. Immunity 67, 5417 - 5426). The epitope recognised by MAb B5 was identified in all LPS immunotypes with phosphoethanolamine (PEtn) in the 3-position of ß-chain heptose (HepII) of inner core LPS. Further work was carried out to identify additional epitopes, with the aims outlined in Fig 4

In summary:
A series of twelve murine monoclonal antibodies (MAbs) were developed at NRC, by using a procedure described previously by us (Plested *et al.*, 1999 Infect. immunity 67, 5417 - 5426), except using formalin-fixed *Neisseria meningitidis* L4 (strain 891) *galE* whole-cells. The twelve MAbs were extensively screened by ELISA using purified LPS from *Neisseria meningitidis* mutants and wild-type strains and three MAbs B2 (IgG2b), A4 (IgG2a), and A2 IgG2a were chosen for further investigation. Conservation of the inner core LPS epitope was assessed at Oxford using wild-type whole-cell lysates of a global collection of 104 *Neisseria meningitidis* disease isolates (Maiden, M.C.J., J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, I.M. Feavers, M. Achtman, and G.B. Spratt. 1998. PNAS 95: 3140-3145). Accessibility of the inner core LPS epitope was assessed using immunofluorescence microscopy with ethanol-fixed *Neisseria meningitidis* whole-cells of wild type and mutants adherent to a monolayer epithelial cells (Plested *et al.* 1999).

Each of the three MAbs reacted with purified *Neisseria meningitidis* L4 *galE* LPS by ELISA. Except for MAb B2 that had low reactivity with *Neisseria meningitidis* L4 LPS, none of the *Neisseria meningitidis* L4 series of MAbs were able to the recognise wild-type L4 or L2 purified LPS by ELISA. None of the *Neisseria meningitidis* L4 MAbs recognised *Neisseria meningitidis* wild-type L2 or L4 whole-cells by immunofluorescence microscopy.

MAb B2 reacted with 15/32 *Neisseria meningitidis* MAb B5 negative *Neisseria meningitidis*

strains and 9/68 *Neisseria meningitidis* MAb B5 positive *Neisseria meningitidis* strains by whole-cell dot blot analysis. MAb 2 reacted with L4 *galE*, L4 wild-type (very low reactivity) but not L3 *galE*, L2 *galE* (native/O-deacylated (odA)), L2 wild-type (native-odA), L5, L6 wild-type LPS.

MAb A2 recognised 28/32 *Neisseria meningitidis* MAb B5 negative *Neisseria meningitidis* strains and 20/68 *Neisseria meningitidis* MAb B5 positive *Neisseria meningitidis* strains by whole-cell dot blot analysis. MAb A2 reacted with L4 *galE* (native/odA), L2 *galE* (native) but not L3 *galE*, L2 *galE* (odA), L2 wild-type (native/odA), L4, L5, L6 wild type LPS.

MAb A4 reacted with 29/32 *Neisseria meningitidis* MAb B5 negative *Neisseria meningitidis* strains and 24/68 *Neisseria meningitidis* MAb B5 positive *Neisseria meningitidis* strains by whole-cell lysate dot blot analysis. MAb A4 reacted with L4 *galE*, L2 *galE* (native/odA), but not L3 *galE*, L2 wild type, L4, L5, L6, L8 wild-type LPS.

Based on these results, MAb A4 (IgG2a) was chosen for further study as it demonstrated specificity for both L4 *galE* and L2 *galE* LPS by ELISA and recognised all except 3 *Neisseria meningitidis* B5 negative *Neisseria meningitidis* strains (BZ232 serogroup B; NGH38 serogroup B; F1576 serogroup C). Together MAbs B5 and A4 were able to recognise 97/100 *Neisseria meningitidis* isolates. Immunofluorescence microscopy demonstrated that MAb A4 was able to access the inner core epitope in an L4 *galE* mutant in the presence of capsule.

We have identified LPS inner-core epitopes with PEtn at the 3-position of HepII (MAb B5) or not at the 3 position (MAb A4). There remain 3 strains out of 100 (BZ232, NGH38 and F1576) which show no reactivity with either MAb A4 or MAb B5. The structural basis for this non-reactivity is under investigation. Once all the variant glycoforms of the inner core are known, of which at least 3 have been identified, the rationale will exist for including epitopes, representative of all *Neisseria meningitidis* strains causing invasive disease, in a conjugate vaccine. This will be tested for proof in principle using studies in animals before proceeding to human trials.

The following techniques were used :

(1) Murine MAb A4 (IgG2a) was raised to *galE* (89I, L4 immunotype) and selected on basis of reactivity in LPS ELISA & immunofluorescence (IF) microscopy.

(2) LPS ELISA (Plested *et al.*, 2000 J. Immunol. Meth. 237:73-84): Microtitre plates (Nunc) coated with purified (*galE*) LPS (10μg/ml) overnight, were washed, blocked, incubated with MAb for 1h, washed and detected with anti-mouse IgG alkaline phosphatase and p-NPP ($OD_{A405nm}$).

(3) Immunoblotting using whole-cell lysates from 104 *Neisseria meningitidis* strains (Plested *et al.*, 1999 IAI 67:5417-5426). MAb A4 was detected using anti-mouse IgG alkaline phosphatase and BCIP/NBT.

(4) Immunofluorescence microscopy: as before (Plested *et al.*, 1999 IAI 67:5417-5426) or *Neisseria meningitidis* were adherent to cultured human buccal epithelial cell line (16HBE140) instead of HUVECs; fixed, blocked, incubated with MAb A4 and anti-capsular serogroup B antibody then detected using fluorescently labelled secondary antibodies (TRITC or FITC).

(5) Fine structural analysis of purified *O*-deacylated LPS samples by negative-ion ES-MS and NMR (Plested *et al.*, 1999 IAI 67:5417-5426).

**RESULTS:**

**1) Accessibility of LPS epitope in *Neisseria meningitidis* whole-cells.**

See Figure 7.

MAb A4 accesses the inner core LPS epitope in *Neisseria meningitidis* L4 *galE* mutant in the presence of capsule (magnification x 100). *Neisseria meningitidis* L4 *galE* adherent to epithelial cells (16HBE140) stained with:

a. MAb A4 (anti-mouse TRITC-red);

b. anti-cap B (anti-rabbit FITC- green);

c. triple staining with MAb A4 (anti-mouse TRITC-red), anti-cap B (anti-rabbit FITC-green) and epithelial cells stained DAPI (blue).

MAb B5 accesses inner core LPS epitopes in *Neisseria meningitidis* L3 MC58 (magnification x 2400). *Neisseria meningitidis* L3 MC58 adherent to HUVECs stained with d. MAb B5 (anti-mouse TRITC-red), e. anti-cap B (anti-rabbit FITC-green) using confocal immunofluorescence microscopy.

**2) Conservation of LPS epitope across all serogroup of *Neisseria meningitidis***

See Figure 8.

MAb A4 (diagonal hatched) and MAb B5 (horizontal lines) together recognise all *Neisseria meningitidis* strains by immunoblotting with whole-cell lysates, except 3 strains (black-arrows) which are under further analysis. The dendrogram of genetic relationship of *Neisseria meningitidis* strains from a global collection was constructed by cluster analysis following Multi-Locus Sequence Typing (MLST) (Maiden *et al.*, 1998 PNAS 95:3140-3145).

**3) Genetically defined LPS structure**

See Figure 3.

Fine LPS structural details demonstrates conformational effects of PEtn on epitope presented. Space-filling 3-D molecular models of (Metropolis Monte Carlo) calculated lowest energy states of core LPS from *galE* mutants a. L3; b. L4; c. L8 (dephosphorylated). Kdo in grey,

Heptose (Hep) in red, Glucose (Glc) and Glucosamine (GlcNAc) in light and darker green, (PEtn) in brown.

**CONCLUSIONS:**

Inner core glycoforms have been identified with PEtn in the 3-position of HepII, an exocyclic position of Hep II or absent. This study has indicated that utilisation of MAb A4 in conjunction with MAb B5 enables 97% of meningococcal strains to be recognised. These studies therefore indicate that inner core LPS may have potential as a *Neisseria meningitidis* serogroup B vaccine.

**Example 3 Studies on the functional activity of monoclonal antibody, MAb B5, and inner core (*galE*) lipopolysaccharide antibodies in human serum using an opsonophagocytosis assay, a serum bactericidal assay and an *in vivo* passive protection model**

**INTRODUCTION**

We have generated a monoclonal antibody, MAb B5. This antibody is accessible to inner core LPS structures in *Neisseria meningitidis* in the presence of capsule and is conserved in 70% of a representative collection of *Neisseria meningitidis* of all strains and 76% of serogroup B strains (Plested, J.S. *et al.* (1999) Infect. & Immun. 67 (10): 5417-5426).

Until now it was not known if antibodies in a natural human infection can be specific for MAb B5 epitope and have functional activity.

MAb B5 has been shown to have opsonic and bactericidal activity against *galE* mutant and ability to passively protect infant rats against challenge with *Neisseria meningitidis galE* mutant using an *in vivo* model.

## METHODS

(1) **Opsonophagocytosis (OP) assay** (Plested *et al.*, 2000b): Briefly, fluorescently labelled ethanol-fixed *Neisseria meningitidis* MC58 or *galE* mutant or beads coated with purified *galE* LPS (10µg/ml) were opsonised with MAb B5 and human complement source diluted in final buffer for 10mins/37C/ 500rpm in microtitre plate. Then human peripheral blood polymorphonuclear cells (PMNs) prepared from heparinised donor blood were diluted in final buffer and added to each well (1 x $10^7$ cells/ml) and incubated for a further 10min/37°C/ 500rpm. Reaction mixture was stopped on ice by addition of 150µl PBS-EDTA and added to FACS tube containing 50µl Trypan Blue. Mixture was mixed and 10,000 lymphocytes were analysed on FACScan and Cellquest software. PMNs were analysed by FSC vs appropriate channels to determine % uptake of fluorescent bacteria by granulocytes and monocytes (% OP activity).

(2) **Serum Bactericidal (SB) assay** method was adapted from CDC protocol except MAb B5 was added to dilutions of human pooled sera and 1000 cfu of *Neisseria meningitidis* strain and incubation time was 40-45min at 37°C. Briefly, bacteria were grown up onto BHI agar overnight from frozen stocks. A suspension of bacteria in PBS-B was measured at $OD_{260}$ (1:50 in 1% SDS, 0.1% NaOH). Using a 96-well microtitre plate 50µl buffer was added to wells in columns 2-7. 50µl of 80% decomplemented human pooled sera was added to column 8 wells. 100µl of 80% pooled sera was added to wells in column 1. Two-fold serial dilutions of antibody were added to columns 1-7 (discarding the last 50µl from column 7). 50ul of bacterial suspension diluted to give 1000 cfu in 50µl were added to wells of columns 1-8. The mixture was incubated for 40-45 minutes and plated out onto BHI agar for overnight incubation. The number of colonies on each plate were counted and the results expressed as a % of cfu/ml in decomplemented control well.

(3) ***In vivo* passive protection model** using 5-day old Wistar infant rats model. This model was as described by Moe, G.R., *et al.*, 1999. Infect. Immun. 67: 5664-5675, except higher doses of *Neisseria meningitidis* bacteria were used and different *Neisseria meningitidis* strain

was used. Briefly, groups of 5 day old infant rats were randomised with mothers, weighed and given inoculum 1 x $10^8$ cfu/ml *Neisseria meningitidis galE* mutant mixed 1:1 with either (i) No antibody (PBS) (ii) Affinity purified MAb B5 (10µg) (iii) Affinity purified MAb B5 (100µg) (iv) MAb 735 (anticapsular group B antibody) (2µg). Infant rats were monitored for signs of infection and sampled by tail vein bleed at 6 hours post-infection. Animals were weighed and terminal bleed was taken after 24h by cardiac puncture following injection of pentobarbitone. Neat and diluted blood were plated immediately onto BHI plates and incubated overnight. Plates were counted next day to determine bacteremia (cfu/ml) at 6h and 24h.

(4) **LPS ELISA** (Plested *et al.*, 2000a. Microtitre plates (Nunc) coated with purified (*galE*) LPS (10µg/ml) overnight, were washed, blocked and incubated with MAb or human sera for 1h, washed and detected with anti-mouse or anti-human IgG alkaline phosphatase and p-NPP ($OD_{A405nm}$).

(5) **Affinity purified MAb B5.** Spent culture supernatant from MAb B5 was purified on Protein A–sepharose column and eluted with Glycine pH 4.0, neutralised with Tris-HCl pH 9.0. Fractions were tested for reactivity on LPS ELISA, pooled and concentrated using Amicon-filter. Purity was determined by SDS-PAGE gel and protein concentration was determined by OD and protein assay.

(6) **FACS surface labelling of *Neisseria meningitidis* bacteria**

The method was adapted from Moe *et al.* (Moe, G.R., *et al.*, 1999. Infect. Immun. 67: 5664-5675) except no sodium azide was included in the blocking buffer step (Plested *et al.*, 2000b. To prepare labelled bacteria *Neisseria meningitidis* (strain MC58, *galE*) organisms were grown overnight by standard conditions at 37°C on BHI agar plates and gently suspended in PBS. $OD_{A260nm}$ was adjusted to give the required concentration e.g. 5 x $10^9$ org./ml. 100µl bacterial cells were added to each FACS tube (5 x $10^8$ org.) and an equal volume of diluted sera (1/100 MAb B5 in 1% BSA/PBS) was added. Tubes were incubated for 2 hours at 4°C and cells centrifuged for 5 minutes at 13,000 g. The supernatant was discarded and cells were washed with 200µl of 1% BSA/PBS. 100µl of FITC-conjugated $F(ab)_2$ goat anti-mouse

(Sigma F2772) was added, diluted 1:100 in 1% BSA/PBS, and tubes were incubated for 1 hour at 4°C. Cells were centrifuged at 13,000 *g* for 5 minutes and washed by addition of 200µl of 1% BSA/PBS. The supernatant was discarded and the cells were suspended in 1% v/v formaldehyde. Samples were transferred to FACScan tubes and analysed on the FACS.

## RESULTS

### 1) Clinical relevance of MAb B5 epitope:

We present data on three paired sera taken from infants early (acute) and later (convalescent) during culture confirmed invasive meningococcal disease (IMD) that resulted from infection with *Neisseria meningitidis* isolates of immunotypes L1, L3 (MAb B5 reactive) (patients 1 and 2) and L2 immunotype (MAb B5 non-reactive) (patient 3) (Figure 10). The *Neisseria meningitidis* isolates for patients 1, 2, 3 were L1 (B nt p1.14), L3 (B15 p1.7) and L2 (C2a p1.5) respectively. One paired sera from patient 2 infected with a *Neisseria meningitidis* strain that was MAb B5 reactive demonstrated an increase in specific inner core LPS antibodies by ELISA between early and late infection ($p=0.03$ not significant two-tailed paired t-test, 95% CI 0.09-90.8)) (Figure 10a). Patient 1 sera demonstrated no significant difference in the titre of antibody taken early and later during IMD but the titre of the early sample was already at a high level (Figure 10a). The lack of increase may reflect higher affinity antibody in the convalescent sample that would not be detected in this ELISA. However in both patient 1 and 2 sera there was a nearly significant increase in functional activity in the convalescent sera in an opsonophagocytosis assay with L3 wild-type strain MC58 and human peripheral polymorphonuclear cells ($p=0.06$ two-tailed paired t-test, 95%CI 0.90-5.96) (Figure 10b) (Plested *et al.*, 2000b). There was no significant increase in specific antibody titre between acute and convalescent sera taken from patient 3 infected with L2 immunotype strain (MAb B5 non-reactive) as measured by ELISA (Figure 10a). There was no significant functional activity in OP assay against L3 wild-type strain with sera taken from patient 3 early or later during IMD (Figure 10b). This demonstrates the clinical relevance of the MAb B5 epitope *in vivo* and that specific inner core LPS antibodies are functional *in vivo*.

Figure 10. A. ELISA titres of antibodies to L3 *galE* LPS (IgG) in paired sera taken early and late from children with invasive meningococcal disease.
B. Mean % phagocytosis of *Neisseria meningitidis* MC58 with paired sera taken early and late from children with invasive meningococcal disease with human peripheral blood mononuclear cells and human complement.

**2) Supporting evidence that murine MAb B5 has functional activity in biologically relevant assays and an *in vivo* model.**

**(i) Opsonophagocytosis assay**

The OP assay provides evidence that MAb B5 has opsonic activity against *Neisseria meningitidis* wild type and *galE* mutant and that the OP activity is specific for MAb B5 epitope.

The specificity of MAb B5 reactivity using wild-type *Neisseria meningitidis* MC58 was shown by inhibition studies. MAb B5 was pre-incubated with different concentrations of purified LPS. There was a dose response inhibition in OP activity with *Neisseria meningitidis* MC58 with increasing concentrations of *galE* LPS added to MAb B5 (see Figure 11a).

Figure 11a. Mean % phagocytosis of *Neisseria meningitidis* MC58 with MAb B5 pre-incubated with increasing concentrations of either (i) B5 reactive or (ii) B5 non-reactive *galE* LPS with human peripheral blood polymorphonuclear cells and human complement.

MAb B5 has specific OP activity for MAb B5 reactive strains using an isogenic pair of *Neisseria meningitidis* wild-type strains (*Neisseria meningitidis* BZ157, serogroup B) that are MAb B5 reactive or MAb B5 non-reactive. MAb B5 has opsonic activity with MAb B5 reactive strain but not MAb B5 non-reactive strain (see Figure 11b).

Figure 11b. Mean % phagocytosis of pair of *Neisseria meningitidis* wild-type isogenic strains (*Neisseria meningitidis* BZ157) that are either MAb B5 reactive or B5 non-reactive with MAb B5 as the opsonin with human peripheral blood mononuclear cells and human complement.

OP assay demonstrated the uptake of beads coated with purified L3 *galE* LPS opsonised with MAb B5 was significantly greater than the uptake with uncoated beads. This demonstrates the specificity of MAb B5 for *galE* LPS coated onto beads (see Figure 11c).

Figure 11c. Mean % phagocytosis of fluorescent latex beads coated with either purified LPS from L3 *galE* mutant (10µg/ml) or uncoated, in the presence of MAb B5 or final buffer, with human peripheral blood mononuclear cells and human complement.

**(ii) Serum bactericidal assay**

The SB assay provides evidence that MAb B5 has bactericidal activity against *Neisseria meningitidis galE* mutant in SB assay in the presence of a human complement source (see method).

The serum sensitivity of *galE* mutant with either no antibody or in the presence of MAb B5 was compared (Figure 12). There was a dose response increase in bactericidal activity of *galE* mutant shown by decreasing % survival, with decreasing % of serum in the presence of MAb B5 compared to no antibody.

Figure 12. Mean % survival of *Neisseria meningitidis galE* mutant in the presence and absence of MAb B5 against two-fold serial dilutions of human pooled serum starting at 40% as detected using a serum bactericidal assay (see methods).

**(iii) Passive protection model using the infant rat.**

Using the 5-day-old infant rat model we have demonstrated that two doses MAb B5 are able to reduce bacteremia against challenge with 1 x $10^8$ cfu/ml *Neisseria meningitidis* MC58 *galE* mutant i.p. compared to no antibody controls. This data demonstrates the ability of MAb B5 to

passively protect against challenge with *Neisseria meningitidis* MC58 *galE* mutant and correlates with the functional activity of MAb B5 in OP and SB assays against the same *Neisseria meningitidis* strain.

Figure 13. Geometric mean bacteremia in the blood of groups of 5 day old infant rats 24h post-infection with 1 x $10^8$ cfu/ml *galE* mutant given simultaneously with either: (i) no antibody (ii) MAb B5 (10µg dose); (iii) MAb B5 (100µg dose); (iv) MAb 735, a positive control anti-capsular antibody (2µg dose).

**MAb B5 binding studies**

Additional evidence that MAb B5 recognises both wild-type and *galE* mutant LPS is shown in the following binding studies:

**a) Western blot analysis**

Purified LPS from wild type *Neisseria meningitidis* MC58 and *galE* mutant was separated on standard Tricine gel and blotted onto nitrocellulose by standard methods. The blot was probed with MAb B5 culture ascites (1:2000) overnight and detected using anti-mouse IgG and BCIP/NBT substrate. The blot demonstrates binding of MAb B5 to higher molecular weight wild-type LPS band and lower molecular weight *galE* LPS band in wild-type LPS. This demonstrates that MAb B5 can access and bind to the wild-type LPS as well as truncated *galE* LPS.

Figure 14. Western blot showing purified LPS from *Neisseria meningitidis* MC58 and *galE* mutant probed with MAb B5 (ascites fluid 1:2000) detected using anti-mouse IgG alkaline phosphatase and BCIP/NBT substrate.

**b) FACS surface labelling data**

MAb B5 binding to live wild-type strain MC58 and *galE* mutant (1 x 108 cfu/ml) were quantitatively compared using surface labelling with anti-mouse FITC and analysed by FACS.

The relative binding of MAb B5 to *Neisseria meningitidis* MC58 was 82.5% and *Neisseria meningitidis galE* mutant was 96.9% demonstrating that as expected the greatest binding was to the *galE* mutant but there was still significant binding to the wild-type strain MC58.

Figure 15. FACS profile comparing surface labelling of live *Neisseria meningitidis* MC58 and *galE* mutant (5 x 108 org./ml) with MAb B5 (culture supernatant 1:50) detected using anti-mouse IgG (FITC labelled).

**Claims**

1. A vaccine for the treatment of disease caused by pathogenic *Neisseria*, the vaccine comprising an immunogenic component based on the inner core of a *Neisseria* lipopolysaccharide, LPS, and being capable of eliciting functional antibodies against a majority of the strains within the species of the pathogenic *Neisseria*.

2. A vaccine according to claim 1, wherein the said immunogenic component is capable of eliciting functional antibodies against at least 60% of the strains within the species of the pathogenic *Neisseria*.

3. A vaccine according to claim 2, wherein the said immunogenic component is capable of eliciting functional antibodies against at least 70% of the strains within the species of the pathogenic *Neisseria*.

4. A vaccine according to any preceding claim, wherein the immunogenic component is substantially free from outer core lipopolysaccharide.

5. A vaccine according to any preceding claim, wherein the species of the pathogenic *Neisseria* is *Neisseria meningitidis*.

6. A vaccine according to claim 5, wherein the antibodies are elicited by the immunogenic component in at least 50 % of group B strains of *Neisseria meningitidis*.

7. A vaccine according to claim 5, wherein the antibodies are elicited by the immunogenic component in at least 60% of group B strains of *Neisseria meningitidis*.

8. A vaccine according to claim 5, wherein the antibodies are elicited by the immunogenic component in at least 70% of group B strains of *Neisseria meningitidis*.

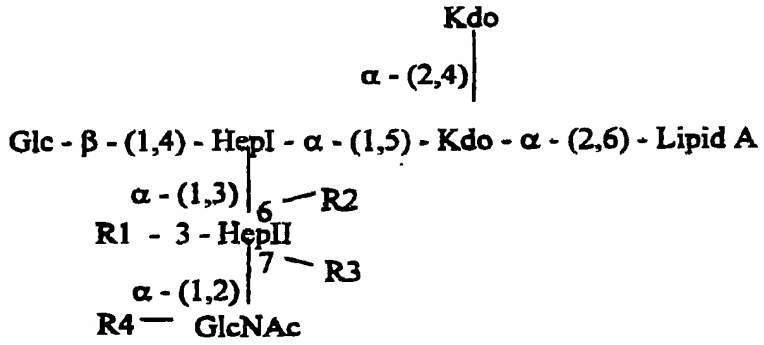
9. A vaccine according to any preceding claim, wherein the immunogenic component comprises of or consists of an epitope which is a part or all of the inner core structure of a *Neisseria* LPS, is derived from this inner core, is a synthetic version of the inner core, or is a functional equivalent thereof.

10. A vaccine according to any preceding claim, wherein the immunogenic component is an epitope on the LPS inner core characterised by the presence of a phosphoethanolamine moiety linked to the 3-position at HepII of the inner core, or is a functional equivalent thereof.

11. A vaccine according to any preceding claim, wherein the immunogenic component is an epitope on the LPS inner core which comprises a glucose residue at HepI.

12. A vaccine according to any preceding claim, wherein the immunogenic component is an epitope on the LPS inner core which comprises an N-acetyl glucosamine at HepII of the inner core LPS.

13. A vaccine according to any preceding claim, wherein the inner core LPS consists of an inner core oligosaccharide attached to lipid A, with the general formula as shown:




where R1 is a substituent at the 3-position of HepII, and is hydrogen or Glc-α-(1, or phosphoethanolamine; R2 is a substituent at the 6-position of HepII, and is hydrogen or phosphoethanolamine; R3 is a substituent at the 7-position of HepII, and is hydrogen or

phosphoethanolamine, and R4 is acetyl or hydrogen at the 3-position, 4-position or 6-position of the GlcNAc residue, or any combination thereof; and where Glc is D-glucopyranose; Kdo is 3-deoxy-D-*manno*-2-octulosonic acid; Hep is L-*glycero*-D-*manno*-heptose, and GlcNAc is 2-acetamido-2-deoxy-D-glucopyranose.

14. A vaccine according to any preceding claim, wherein the immunogenic component is reactive with the B5 antibody produced by the hybridoma deposited under accession number IDAC 260900-1.

15. A vaccine comprising a few immunogenic components based on the inner core of a *Neisseria* lipopolysaccharide, LPS, and being capable of eliciting functional antibodies against a majority of the strains within the species of the pathogenic *Neisseria*.

16. A vaccine according to claim 15 and including an immunogenic component as defined in any of claims 1 to 14.

17. A vaccine according to claim 15 or 16, wherein the said few immunogenic components elicit functional antibodies in at least 85% of the strains within the species of the pathogenic *Neisseria*.

18. A vaccine according to claim 17, wherein the said few immunogenic components elicit functional antibodies in at least 95% of the strains within the species of the pathogenic *Neisseria*.

19. A vaccine according to any of claims 15 to 18, wherein an immunogenic component is reactive with the A4 antibody produced by the hybridoma deposited under accession number IDAC 260900-2.

20. A vaccine according to any preceding claim, wherein the immunogenic element of the vaccine is an epitope accessible on the bacterium in the presence of bacterial capsule.

21. A vaccine according to any preceding claim, comprising one or more immunogen components which are capable of stimulating antibodies which are opsonic.

22. A vaccine according to any preceding claim for the treatment of *Neisseria meningitidis*.

23. A vaccine according to claim 22 for the treatment of *Neisseria meningitidis* group B.

24. A vaccine according to any preceding claim for the prevention of meningitis, septicaemia or pneumonia or other manifestation of systemic or local disease occasioned by *Neisseria meningitidis*.

25. A vaccine according to any of claims 1 to 22 for the treatment of urethritis, salpingitis, cervicitis, proctitis, pharyngitis, pelvic inflammatory disease or other manifestation of systemic or local disease occasioned by *Neisseria gonorrhoeae*.

26. A vaccine according to any preceding claim which is a conjugated vaccine.

27. A vaccine according to any preceding claim, which is derived from a commensal *Neisseria*.

28. A vaccine according to claim 27, wherein the commensal *Neisseria* is *Neisseria lactamica*.

29. An antibody reactive with an immunogenic component as defined in any preceding claim.

30. An antibody according to claim 29, wherein the antibody is humanized or otherwise customised to enhance suitability for administration to a human.

31. An antibody according to claim 29, obtainable from the hybridoma producing antibody B5.

32. An antibody according to claim 29, obtainable from the hybridoma producing antibody A4.

33. A hybridoma producing antibody B5.

34. A hybridoma producing antibody A4.

35. A pharmaceutical preparation comprising an antibody according to any of claims 29 to 32 in combination with a pharmaceutically acceptable carrier.

36. A method for the treatment of *Neisseria* infection, the method comprising administering to a subject in need of such treatment an effective amount of a vaccine according to any of claim 1 to 28.

37. A method for the treatment of *Neisseria* infection, the method comprising administering to a subject in need of such treatment an effective amount of an antibody according to any of claims 28 to 31.

38. A method for the identification of immunogenic epitopes of strains of a species of *Neisseria*, the method comprising the steps of generating antibodies to the inner core of a *Neisseria* bacterium, by inoculation of a host organism with a *galE* mutant strain of *Neisseria meningitidis*, and testing such antibodies against a wild type *Neisseria meningitidis* strain to identify those antibodies which are reactive, and for which the epitopes are therefore accessible.

39. Use of one or more biosynthetic pathway genes in the production of a *Neisseria* strain for the assessment, treatment or prevention of *Neisseria* infection.

40. Use of an immunogenic component, or a few immunogenic components, based on the inner core of a *Neisseria* lipopolysaccharide, LPS, and being capable of eliciting

functional antibodies against a majority of the strains within the species of the pathogenic *Neisseria*, in the preparation of a medicament for the treatment of a disease caused by a pathogenic *Neisseria* infection.

41. Use of an antibody according to any of claims 29 to 32 in the preparation of a medicament for the treatment of *Neisseria* infection.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

# PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>12 June 2001 (12.06.01) | |
| **International application No.**<br>PCT/GB00/03758 | **Applicant's or agent's file reference**<br>WPP82704 |
| **International filing date** (day/month/year)<br>02 October 2000 (02.10.00) | **Priority date** (day/month/year)<br>30 September 1999 (30.09.99) |
| **Applicant**<br>PLESTED, Joyce, Susan et al | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 April 2001 (26.04.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|---|---|
| **The International Bureau of WIPO**<br>**34, chemin des Colombettes**<br>**1211 Geneva 20, Switzerland** | Authorized officer<br>Olivia TEFY |
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

WEST

Generate Collection Print

L2: Entry 1 of 2 File: JPAB Nov 24, 1999

PUB-NO: JP411322793A
DOCUMENT-IDENTIFIER: JP 11322793 A
TITLE: ANTIGEN ZYGOTE CONSISTING OF PRESERVATIVE LIPOPOLYSACCHARIDE OF GRAM NEGATIVE BACTERIUM

PUBN-DATE: November 24, 1999

INVENTOR-INFORMATION:

| NAME | COUNTRY |
|---|---|
| ARUMUGHAM, RASAPPA G | |
| FORTUNA-NEVIN, MARIA | |
| APICELLA, MICHAEL A | |
| GIBSON, BRADFORD W | |

ASSIGNEE-INFORMATION:

| NAME | COUNTRY |
|---|---|
| AMERICAN CYANAMID CO | |

APPL-NO: JP11061354
APPL-DATE: March 9, 1999

INT-CL (IPC): C07 K 14/34; A61 K 31/00; A61 K 39/02; A61 K 39/08; A61 K 39/09; A61 K 39/10; A61 K 39/104; A61 K 39/385

ABSTRACT:

PROBLEM TO BE SOLVED: To obtain an antigen zygote inducing not only an immunogenic response against a specified species of a gram negative bacterium but also a cross reaction immune response against a different strain or a different serum type from that of a gram negative bacterium belonging to a specified genus, preferably against a gram negative bacterium of a different genus, and a vaccine containing the antigen zygote.

SOLUTION: The antigen zygote comprising a carrier protein bound by a covalent bond to a preservative part consisting of an inside core part and a lipid A part of lipopolysaccharide of a gram negative bacterium and a vaccine containing the antigen zygote. This antigen zygote induces a cross reaction immune response against a different strain of the gram negative bacterium, and preferably, against a gram negative bacterium of a different genus.

(19)日本国特許庁（ＪＰ）　(12)公開特許公報（A）　(11)特許出願公開番号

特開平11－322793

(43)公開日　平成11年(1999)11月24日

| (51)Int.Cl.[6] | 識別記号 | ＦＩ | |
|---|---|---|---|
| Ｃ０７Ｋ　14/34 | | Ｃ０７Ｋ　14/34 | |
| Ａ６１Ｋ　31/00 | ６３７ | Ａ６１Ｋ　31/00 | ６３７Ａ |
| 39/02 | | 39/02 | |
| 39/08 | | 39/08 | |
| 39/09 | | 39/09 | |

審査請求　未請求　請求項の数18　ＯＬ　（全 18 頁）　最終頁に続く

(21)出願番号　特願平11－61354

(22)出願日　平成11年(1999)３月９日

(31)優先権主張番号　０９／０３７５２９

(32)優先日　1998年３月10日

(33)優先権主張国　米国（ＵＳ）

(71)出願人　591000791
アメリカン・サイアナミド・カンパニー
AMERICAN CYANAMID COMPANY
アメリカ合衆国ニュージャージイ州07940－0874　マディソン・フアイブジラルダフアームス（番地なし）

(72)発明者　ラサッパ・ジー・アルムガム
アメリカ合衆国14534ニューヨーク州エラティア・サークル・ピッツフォード15番

(74)代理人　弁理士　青山　葆　（外１名）

最終頁に続く

(54)【発明の名称】　グラム陰性菌の保存リポ多糖からなる抗原接合体

(57)【要約】

【課題】　グラム陰性菌の所定の種に対する免疫原性応答だけでなく、所定の属に属するグラム陰性菌の異なる株または血清型や、好ましくは異なる属のグラム陰性菌に対する交差反応免疫応答を誘発する抗原接合体およびそれを含有するワクチンを提供すること。

【解決手段】　グラム陰性菌のリポ多糖の内側コア部分とリピドＡ部分とからなる保存部分に共有結合した担体タンパクからなる抗原接合体およびそれを含有するワクチン。この抗原接合体は、グラム陰性菌の異なる株に対する、および、好ましくは異なる属のグラム陰性菌に対する、交差反応免疫応答を誘発する。

1

【特許請求の範囲】

【請求項1】　グラム陰性菌のリポ多糖の保存部分に共有結合した担体タンパクからなる抗原接合体であって、リポ多糖の該保存部分が該リポ多糖の内側コア部分とリピドA部分とからなり、該接合体が該グラム陰性菌の異なる株に対する交差反応免疫応答を誘発することを特徴とする抗原接合体。

【請求項2】　前記接合体が異なる属のグラム陰性菌に対する交差反応免疫応答を誘発する請求項1記載の抗原接合体。

【請求項3】　前記リポ多糖が脱O-アシル化されている請求項1記載の抗原接合体。

【請求項4】　前記担体タンパクが、破傷風毒素またはトキソイド、ジフテリア毒素またはトキソイド、ジフテリア変異体毒素$CRM_{197}$、シュードモナス属菌(Pseudomonas)の外毒素A、コレラ毒素またはトキソイド、A型レンサ球菌毒素、ストレプトコッカス・ニュモニエ(Streptococcus pneumoniae；肺炎レンサ球菌)の肺炎レンサ球菌溶血毒素、ボルデテラ・ペルツッシス(Bordetella pertussis；百日咳菌)のフィラメント状赤血球凝集素(FHA)またはFHA断片、ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)の線毛またはピリン、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の線毛またはピリン、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の外膜タンパク、ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)の外膜タンパク、ストレプトコッカス属菌(Streptococcus；レンサ球菌)のC５Aペプチダーゼおよびモラクセラ・カタラーリス(Moraxella catarrhalis)の表面タンパクからなる群から選択される請求項1記載の抗原接合体。

【請求項5】　前記担体タンパクが、スルホスクシンイミジル-6-(3-[2-ピリジルジチオ]プロピオンアミド)-ヘキサノエート(スルホ-LC-SPDP)、スクシンイミジル-6-(3-[2-ピリジルジチオ]プロピオンアミド)-ヘキサノエート(LC-SPDP)、トラウト(Traut)試薬(2-イミノチオラン)、N-スクシンイミル-S-アセチルチオアセテート(SATA)、N-スクシンイミジル-3-(2-ピリジルジチオ)プロピオネート(SPDP)、スクシンイミジルアセチルチオプロピオネート(SATP)、スクシンイミジル-4-(N-マレイミドメチル)シクロヘキサン-1-カルボキシレート(SMCC)、マレイミドベンゾイル-N-ヒドロキシスクシンイミドエステル(MBS)、N-スクシンイミジル(4-ヨードアセチル)アミノベンゾエート(SIAB)、スクシンイミジル4-(p-マレイミドフェニル)ブチレート(SMPB)、ブロモ酢酸-N-ヒドロキシスクシンイミド(BANS)エステル、1-エチル-3-(3-ジメチルアミノプロピル)カルボジイミド(EDAC)、アジピン酸ジヒドラジド(ADH)、システアミンおよびジチオビス(スクシンイミジルプロピオネート)(DTSSP)からなる群から選択される化合物により、リポ多糖の前記保存部分に結合している請求項1記載の抗原接合体。

2

【請求項6】　前記グラム陰性菌が、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)、ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)、ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)、型別不能ヘモフィルス・インフルエンゼ(non-typeable Haemophilusinfluenzae；型別不能インフルエンザ菌)、ヘモフィルス・デュクレイイ(Haemophilus ducreyi；軟性下疳菌)、ヘリコバクター・ピロリ(Helicobacter pylori)、エシェリキア・コリ(Escherichia coli；大腸菌)、クラミジア属菌(Chlamydia)、サルモネラ属菌(Salmonella)、サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)、サルモネラ・ミネソタ(Salmonella minnesota)、プロテウス・ミラビリス(Proteus mirabilis)、シュードモナス・エルジノーサ(Pseudomonas aeruginosa；緑膿菌)、モラクセラ・カタラーリス(Moraxella catarrhalis)、ボルデテラ・ペルツッシス(Bordetella pertussis；百日咳菌)、シゲラ属菌(Shigella；赤痢菌)、クレブシエラ属菌(Klebsiella)およびビブリオ・コレレ(Vibrio cholerae；コレラ菌)からなる群から選択される請求項1記載の抗原接合体。

【請求項7】　前記グラム陰性菌がナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)である請求項6記載の抗原接合体。

【請求項8】　長鎖N-スクシンイミジル-3-(2-ピリジルジチオ)-プロピオネートおよびブロモ酢酸-N-ヒドロキシスクシンイミドエステルにより、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のリポ多糖の保存部分に共有結合した担体タンパクのジフテリア毒素$CRM_{197}$からなる抗原接合体であって、リポ多糖の該保存部分が該リポ多糖の内側コア部分とリピドA部分とからなり、該接合体がナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の異なる株に対する交差反応免疫応答を誘発することを特徴とする抗原接合体。

【請求項9】　前記接合体が異なる属のグラム陰性菌に対する交差反応免疫応答を誘発する請求項8記載の抗原接合体。

【請求項10】　請求項1の抗原接合体の有効量を含有するワクチン製剤。

【請求項11】　請求項2の抗原接合体の有効量を含有するワクチン製剤。

【請求項12】　請求項8の抗原接合体の有効量を含有するワクチン製剤。

【請求項13】　請求項9の抗原接合体の有効量を含有するワクチン製剤。

【請求項14】　グラム陰性菌により引き起こされる疾

患を予防するために個体を免疫する方法であって、該方法が該個体に予防上有効量のワクチン製剤を予防接種することからなり、該ワクチン製剤がグラム陰性菌のリポ多糖の保存部分に共有結合した担体タンパクからなる抗原接合体からなり、リポ多糖の該保存部分が該リポ多糖の内側コア部分とリピドA部分とからなり、該接合体が該グラム陰性菌の異なる株に対する交差反応免疫応答を誘発することを特徴とする免疫方法。

【請求項15】　前記ワクチン製剤が、前記個体に、皮内、筋肉内、腹腔内、静脈内、膣内、皮下、眼内、鼻腔内および経口投与からなる群から選択される投与経路で投与される請求項14記載の方法。

【請求項16】　前記ワクチン製剤がさらに生理学的担体およびアジュバントを含有する請求項14記載の方法。

【請求項17】　哺乳動物の細菌性敗血症を予防する方法であって、該方法がグラム陰性菌のリポ多糖の保存部分に共有結合した担体タンパクからなる抗原接合体からなる製剤の有効量を投与することからなり、リポ多糖の該保存部分が該リポ多糖の内側コア部分とリピドA部分とからなり、該接合体が該グラム陰性菌の異なる株に対する交差反応免疫応答を誘発することを特徴とする予防方法。

【請求項18】　哺乳動物の細菌性敗血症を予防する方法であって、該方法がグラム陰性菌のリポ多糖の保存部分に共有結合した担体タンパクからなる抗原接合体からなる製剤の有効量を投与することからなり、リポ多糖の該保存部分が該リポ多糖の内側コア部分とリピドA部分とからなり、該接合体が異なる属のグラム陰性菌に対する交差反応免疫応答を誘発することを特徴とする予防方法。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】本発明は、ある種のグラム陰性菌のリポ多糖の保存部分からなる抗原接合体、および、かかる抗原接合体を含有するワクチンに関する。これらの接合体は、グラム陰性菌の異なる株に対する交差反応性を示す抗体を誘発し、かかる接合体を含有するワクチンは、かかるグラム陰性菌に対して機能的かつ防御的である抗体を誘発する。

【0002】

【従来の技術】リポ多糖(ＬＰＳ)は、グラム陰性菌の表面上に多く集中する主要表面抗原である。ＬＰＳ分子は、(1)エステルおよびアミド結合において、ホスフェート、ホスホエタノールアミン基および長鎖脂肪酸で置換されたグルコサミン二糖からなるリピドA部分；(2)八炭糖ケトデオキシオクタノエート(ＫＤＯ)(1～2個の付加的なＫＤＯ分子および3個までのヘプトース部分で置換されていてもよい)によりリピドA部分に結合した内側コア部分；(3)グルコース、ガラクトース、N-



アセチルグルコサミンおよびN-アセチルガラクトサミンなどのヘキソースからなる外部コア部分；ならびに(4)菌株間で大きく変化する反復オリゴ糖単位からなるO特異鎖から構成されている。これらの反復単位が重合して６０,０００ダルトンを超える構造になることは、珍しいことではない。ＬＰＳ分子は、内側コアの構造が菌株間で十分に保存されているが、菌株間で構造および抗原性のレベルを大きく変化させることができる。サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)ＬＰＳのリピドA内側コアの典型的な構造を図1～3に示す。自然防御抗体の発生の原因である免疫応答は、ＬＰＳのこの領域に対する自然免疫により生じると考えられる。

【０００３】非腸内病原体では、ＬＰＳ構造がO抗原反復単位を欠いている。さらに、かかる病原体には、O抗原反復単位の組立てに対する完全な遺伝機構が存在しないと思われる。このことから、これらＬＰＳ構造をリポオリゴ糖(ＬＯＳ)と呼ぶようになった。かかる病原体におけるＬＰＳ構造とＬＯＳ構造との間には、類似点が存在する。例えば、すべてのＬＰＳ構造およびＬＯＳ構造は、ＫＤＯ結合により、リピドA領域をコアに結合している。ＫＤＯ残基の数は、1個(例えば、ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)およびヘモフィルス・デュクレイイ(Haemophilus ducreyi；軟性下疳菌))から2個(例えば、ナイセリア・メニンジティディス(Neisseria meningitidis；骨膜炎菌)およびナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌))まで変化することができる。最近の研究によれば、分岐状オリゴ糖はコア領域から別々に合成され、全ＬＯＳ構造の組立ては細胞質膜の外側で完成されることが示されている(プレストン(Preston)ら、「ザ・リポオリゴサッカライズ・オブ・パソジェニック・グラム-ネガティブ・バクテリア(The Lipooligosaccharides of Pathogenic Gram-Negative Bacteria；病原性グラム陰性菌のリポオリゴ糖)」、クリティカル・レビューズ・イン・マイクロバイオロジー(Critical Reviews In Microbiology),22:139-180(1996)を参照)。

【０００４】したがって、かかるＬＯＳ構造には、別々の内側および外側コア領域はなく、単一のコア領域が存在する。これら病原体のコア構造は、種により変化することができ、ヘプトース完全非存在下のＫＤＯ(例えば、モラクセラ・カタラーリス(Moraxella catarrhalis))；ジヘプトース構造存在下のＫＤＯ(例えば、ナイセリア・メニンジティディス(Neisseria meningitidis；骨膜炎菌)およびナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌))；またはトリヘプトース構造存在下のＫＤＯ(例えば、ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)およびヘモフィルス・デュクレイイ(Haemophilus ducreyi；軟性下疳菌))からなりうる。ナイセリア属菌(Neisseria)および

ヘモフィルス属菌(Haemophilus)由来のコア構造の例をそれぞれ図4および5に示す。オリゴ糖単位は、ヘプトースの各々から延長することができ、および/または、それらはホスホエタノールアミン基で置換されていることができる。ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)２０１９株(フィリップス(Phillips)ら、「ストラクチュラル・キャラクタライゼーション・オブ・ザ・セル・サーフィス・リポオリゴサッカライズ・フロム・ア・ノン-タイパブル・ストレイン・オブ・ヘモフィルス・インフルエンゼ(Structural Characterization of the Cell Surface Lipooligosaccharides from a Non-Typable Strain of Haemophilus Influenzae；ヘモフィルス・インフルエンゼの型別不能株由来の細胞表面リポオリゴ糖の構造的特性決定)」、バイオケミストリー(Biochemistry),31:4515-4526(1992)を参照)およびナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)１２９１株(ジョン(John)ら、「ザ・ストラクチュラル・ベイシズ・フォー・ピオシン・レジスタンス・イン・ナイセリア・ゴノレエ・リポオリゴサッカライズ(The Structural Bases for Pyocin Resistance in Neisseria gonorrhoeaelipooligosaccharides；ナイセリア・ゴノレエのリポオリゴ糖におけるピオシン抵抗性の構造的基礎)」、ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),266:1903-1911(1991)を参照)の完全なＬＯＳ構造の典型的な例をそれぞれ図6および7に示す。

【０００５】ワクチンの開発におけるＬＰＳの使用は、当該分野で公知である。長い間にわたって、ある特定の病原菌のＬＰＳに対する特異的な抗体応答は、その特定株からの防御に寄与することができると認識されてきた。さらに、糖構造(例えば、ＬＰＳの糖部分)が担体タンパクと接合することができ、かかる接合体を含有するワクチン組成物が所望のＴ細胞依存性応答を誘発することが知られている。この例は、型特異的な莢膜多糖を有する細菌に対する好結果の糖接合体ワクチンである。ワクチン・デザイン(Vaccine Design)：ザ・サブユニット・アンド・アジュバント・アプローチ(The Subunit and Adjuvant Approach)、パウエル，エム・エフ(Powell，M.F.)およびニューマン，エム・ジェイ(Newman，M.J.)，673-694(1995)を参照されたい。この種の免疫応答は、ワクチン・デザイン(Vaccine Design)：ザ・サブユニット・アンド・アジュバント・アプローチ(The Subunit and Adjuvant Approach)、パウエル，エム・エフ(Powell，M.F.)およびニューマン，エム・ジェイ(Newman，M.J.)，695-718(1995)に考察されているように、ヒト乳児における新しい世代の糖-タンパク接合体ワクチンの有効性の基礎である。

【０００６】しかし、かかる病原体の異なる株のＬＰＳは、外側コア糖および/またはＯ特異鎖の広範囲な変化を示すので、単一のＬＰＳを含有するワクチンを利用して、数多くの異なる株または異なる属の細菌に対する抗体応答を発生させる試みは、今日まで成功していない。

【０００７】ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)に対するＬＰＳベースのワクチンを開発する試みにおいては、破傷風トキソイドを、数多くのナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)株のＬＰＳから単離されたオリゴ糖と接合させている(ジェニングズ(Jennings)ら、インフェクション・アンド・イムニティ(Infect. Immun.),43:407-412(1984)を参照)。しかし、この接合体により誘発される抗体は、オリゴ糖特異的であり、高度の血清型特異性を示した。

【０００８】ヴェルヒュール(Verheul)らは、髄膜炎菌ＬＰＳのオリゴ糖と破傷風トキソイドまたは骨膜炎菌外膜タンパクとの接合を開示している〔インフェクション・アンド・イムニティ(Infect. Immun.),61:187-196(1993)〕。マウスにおいて、破傷風トキソイド接合体は、骨膜炎菌に対して殺菌性ではないオリゴ糖特異的な抗体を誘発した。外膜タンパク-ＬＰＳ接合体は、外膜タンパクに対する抗体を誘発したが、ＬＰＳに対する抗体を誘発しなかった。ヴェルヒュール(Verheul)らは、ウサギにおける、数種のナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)株のオリゴ糖と破傷風トキソイドとの接合体の免疫原性についても研究した〔インフェクション・アンド・イムニティ(Infect. Immun.),59:843-851(1991)〕。その結果は、誘発された抗体が、免疫型特異的なエピトープを含有するＬＰＳのオリゴ糖部分にのみ向けられていることを示した。

【０００９】ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の実験室用野生株Ａ１のＬＰＳからオリゴ糖を調製し、引き続いて、担体タンパクとしての破傷風トキソイドと接合させることは、グー(Gu)ら、インフェクション・アンド・イムニティ(Infect. Immun.),61:1873-1880(1993)に開示された。これらの接合体は、マウスおよびウサギにおいて免疫原性であり、抗体の大部分は、免疫型特異的なＬＰＳエピトープに向けられていた。また、接合体抗血清は、ＬＰＳ抗血清ほど、ＬＰＳの様々な免疫型に対する交差反応性を示さなかった。これらの研究は、ＬＰＳから誘導されたオリゴ糖接合体が特異的なオリゴ糖免疫型に対する抗体を誘発することを示す。しかし、この接合体が大部分のナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)株に存在する共通のコア構造に対する有意な交差反応抗体を誘発するという証拠は存在しなかった。

【００１０】バータッチャルジー(Bhattacharjee)らは、エシェリキア・コリ(Escherichiacoli；大腸菌)由来のＬＰＳと、Ｂ型ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の外膜タンパクとの混合物が、接合していない非共有結合型の複合体を形成

することを開示した〔ジャーナル・オブ・インフェクシャス・ディジージズ(J. Infect. Dis.)173:1157-1163(1996)〕。これらの複合体は、数多くのグラム陰性菌と交差反応する抗体を誘発することが見い出された。しかし、これらの複合体が、その後の用量の投与により追加刺激可能なT細胞依存性抗体応答を誘発することができないことが知られている非接合型の糖構造を有する他の調製物と異なる性質を有することを示す証拠は提供されなかった。さらに、かかる非接合型の糖は、乳児において適当な免疫応答を誘発しないことが知られている。

【００１１】グー(Gu)らは、型別不能ヘモフィルス・インフルエンゼ(non-typeable Haemophilus influenzae；型別不能インフルエンザ菌)由来のオリゴ糖と破傷風トキソイドとの接合体の調製を開示している〔インフェクション・アンド・イムニティ(Infect. Immun.),64:4047-4053(1996)〕。しかし、ウサギにおいて誘発された抗血清は、同じ株に対してのみ殺菌活性を示した。

【００１２】

【発明が解決しようとする課題】したがって、グラム陰性菌の所定の種に対する免疫原性応答、好ましくはT細胞依存性応答を効果的に誘発するだけでなく、所定の属に属するグラム陰性菌の異なる株または血清型に対する効果的な交差反応性を示す、抗原接合体、および、かかる接合体を含有するワクチンが依然として必要とされている。さらに、かかる接合体およびワクチンは、異なる属のグラム陰性菌に対する交差反応性を示す抗体を誘発するという利点を有するであろう。

【００１３】

【課題を解決するための手段】本発明は、グラム陰性菌の保存ＬＰＳ部分に共有結合した担体タンパクからなる抗原接合体に関する。ここで、ＬＰＳの保存部分は、少なくともＬＰＳの保存内側コアおよびリピドＡ部分からなる。これらの接合体は、上記グラム陰性菌の異なる株に対する、および、好ましくは異なる属のグラム陰性菌に対する、交差反応免疫応答を誘発する。

【００１４】さらに、本発明は、これらの抗原接合体からなるワクチン、および、グラム陰性菌により引き起こされる様々な疾患を予防するためにかかるワクチンで個体を免疫する方法に関する。

【００１５】

【発明の実施の形態】本発明は、担体タンパクとグラム陰性菌の保存ＬＰＳとの抗原接合体、および、かかる複合体を含有するワクチンに関する。本発明の接合体は、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)、ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)、ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)、型別不能ヘモフィルス・インフルエンゼ(non-typeable Haemophilus influenzae；型別不能インフルエンザ菌)、ヘモフィルス・デュクレイイ(Haemophilus ducreyi；軟性下疳



菌)、ヘリコバクター・ピロリ(Helicobacter pylori)、エシェリキア・コリ(Escherichia coli；大腸菌)、クラミジア属菌(Chlamydia)、サルモネラ属菌(Salmonella)、サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)、サルモネラ・ミネソタ(Salmonella minnesota)、プロテウス・ミラビリス(Proteus mirabilis)、シュードモナス・エルジノーサ(Pseudomonas aeruginosa；緑膿菌)、モラクセラ・カタラーリス(Moraxella catarrhalis)、ボルデテラ・ペルツッシス(Bordetella pertussis；百日咳菌)、シゲラ属菌(Shigella；赤痢菌)、クレブシエラ属菌(Klebsiella)およびビブリオ・コレレ(Vibrio cholerae；コレラ菌)を含めて(ただし、これらに限定されることはない)、様々なグラム陰性菌のＬＰＳを利用している。

【００１６】本発明の接合体、および、これらの接合体を含有するワクチンは、これらの接合体に含有される保存ＬＰＳ部分に対する機能的なポリクローナル抗体応答を生じさせる。かくして、これらのワクチンは、非常に数多くの異なる株の病原体と反応し、それにより、グラム陰性菌の様々な株に対する交差反応性かつ交差機能的な抗体応答を誘発することができる。この交差反応免疫応答は、所定の属に属する異なる株に対してだけでなく、異なる属のグラム陰性菌に対しても示される。

【００１７】かくして、本発明は、グラム陰性菌のＬＰＳの共通保存部分、すなわち数多くのグラム陰性菌に共通するＬＰＳの部分に対する抗体応答を生じる抗原接合体に関する。ここで用いられている「ＬＰＳ」という用語は、スムーズ型ＬＰＳおよびＬＯＳ(あるいは、「ラフ型ＬＰＳ」として知られる)の両方を包含するものとする。

【００１８】上記のように、グラム陰性菌のＬＰＳは、内側コア部分、リピドＡ部分、外側コア部分およびＯ特異的抗原からなる。異なる株または属の細菌に対する応答を誘発するためには、内側コア部分およびリピドＡ部分の構造が保存されていて、本発明の接合体に利用されなければならない。したがって、ここで用いられている、ＬＰＳの「保存部分」という用語は、少なくとも、エステルおよびアミド結合において、ホスフェート、ホスホエタノールアミン基および長鎖脂肪酸で置換されたグルコサミン二糖(すなわち、リピドＡ部分)；ならびに、もしあるなら、内側コア置換基およびヘプトース置換基のＫＤＯ機能を包含するものとする。内側コアに含有されていてもよいホスフェート、ホスホエタノールアミン基およびピロホスホエタノールアミン基は、「保存部分」に含有されていてもよいが、それらは必ずしも必要ではない。「保存部分」に含有される病原体の部分は、菌株の間で高度に保存されており、かくして、これらの構造から広範囲の交差反応抗体が生じることができる(例えば、アピセラ(Apicella)ら、「ザ・ノーマル・ヒューマン・セラム・バクテリサイダル・アンチボディ・レスポンス・トゥ・ザ・リポオリゴサッカライド・オブ・ナイ

セリア・ゴノレエ(The Normal Human Serum Bactericidal Antibody Response to the Lipooligosaccaride of Neisseria gonorrhoeae；ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)のリポオリゴ糖に対する正常なヒト血清殺菌性抗体応答)」、ジャーナル・オブ・インフェクシャス・ディジージズ(J. Infect. Dis.),153:520-528(1986)を参照)。

【００１９】さらに、本発明の範囲内では、ＬＯＳ所有株の内側コアの付加的な分岐状炭化水素、または、ＬＯＳ所有株のオリゴ糖が、ここで定義されている「保存部分」の一部として保存されていてもよい。しかし、これは多くの場合に必要とされないし、望ましいというわけでもない。

【００２０】本発明者らにより、ＬＰＳ構造のかかる保存部分を利用する接合体の生成が、処理されている個体において追加刺激可能なＴ細胞依存性ＩｇＧ応答を誘発すること、および、得られた抗体が、ある特定の菌属内の異なる株の表面だけでなく、異なる属のグラム陰性菌にも交差反応することが見い出された。さらに、本発明の接合体により誘発される表面反応性抗体は、殺菌性(すなわち、免疫防御に関連した機能的な性質を破壊すること)かつ防御的であることが見い出されている。

【００２１】本発明の接合体に利用されるＬＰＳの保存部分は、当業者に公知の数多くの手法で調製しうる。例えば、保存構造は、(1)保存コア構造の全部または一部の化学合成、(2)保存構造を主として含有するＬＰＳを産生する野生型株の選択、(3)野生型株により合成されたＬＰＳの非還元糖残基の酵素分解、ならびに(4)ＰＣＴ出願公開第WO97/19688号に開示されているような、様々な変異体およびそれらの変異体から誘導される子孫による所定の細菌のＬＰＳの保存構造の合成(例えば、ＬＰＳの生合成を欠いた変異株によるナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)ＬＰＳの保存コア部分の製造；バクテリオファージに曝露することによるサルモネラ属菌(Salmonella)またはエシェリキア・コリ(Escherichia coli；大腸菌)のコア欠失ラフ型変異体の製造；野生型株をピオシンに曝露することによるナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)ＬＰＳ変異体およびこれらの変異体から誘導される子孫の製造；ＬＰＳの生合成に関与する特異的酵素のトランスポゾン誘発変異体ならびにＬＰＳの生合成に関与する特異的酵素の部位特異的変異体およびそれらの変異株から誘導される子孫の生成)により調製すればよい。

【００２２】本発明の接合体に用いるためのＬＰＳの保存部分を調製する好ましい手段は、変異体菌株およびそれらの子孫によるＬＰＳ保存部分の合成によるものである(上記の経路4)。さらに好ましくは、下記の変異株を利用して、ＬＰＳ保存部分を合成する。例えば、サルモネラ属菌(Salmonella)のRaコア(内側コアおよび外側コアの両方)、サルモネラ属菌(Salmonella)のRcおよびReならびにエシェリキア・コリ(Escherichia coli；大腸菌)のJ5などの、ＬＰＳの保存コア糖のみを表現する微生物；ＬＰＳのコア部分にグルコースを付加しない微生物；バイオケミストリー(Biochemistry),35:5837-5947(1996)に記載のＢ型ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)由来の変異株２８１.２５などの、ＬＰＳのコア部分にガラクトースを付加しない微生物；ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),269:11162-11169(1994)に記載のナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のＮＭＢ Ｒ６株およびナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)の１２９１-Ｒ６株などの、ホスホグルコムターゼ(ＰＧＭ)遺伝子の変異によりＬＰＳのコア部分にグルコースを付加しない微生物；インフェクション・アンド・イムニティ(Infect. Immun.),63:2508-2515(1995)に記載のナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のＳＳ３変異株などの、ガラクトース・エピメラーゼ(ＧａｌＥ)遺伝子の変異によりＬＰＳのコア部分にガラクトースを付加しない微生物；ならびに、ジャーナル・オブ・エクスペリメンタル・メディスン(J. Exp. Med.),180:2181-2190(1994)に記載のグルコシルまたはガラクトシル・トランスフェラーゼ遺伝子の変異などの対応するトランスフェラーゼ酵素の変異によりＬＰＳのコア部分にグルコースまたはガラクトースを付加しない微生物。

【００２３】リー(Lee)らの研究は、ガラクトース・エピメラーゼが変異すると、グルコースを含有する外側コア部分または分岐部分の後で切断されるＬＰＳが発現することを示している[インフェクション・アンド・イムニティ(Infect. Immun.),63:2508-2515(1995)]。Ｂ群ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のＴｎ９１６生成変異体のスクリーニングは、安定なＬＰＳ変異株(ＮＭＢ-Ｒ６株と呼ばれる)が、$GlcNAc\text{-}Hep_2(PEA)\text{-}KDO_2$-リピドＡという構造を有するディープコアＬＰＳを発現することを示した。さらなる研究は、トランスポゾンが推定上のホスホグルコムターゼ遺伝子に挿入されることを示している(ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),269:11162-11169(1994)を参照)。変異株の細胞非含有抽出物は、ホスホグルコムターゼ活性を示さなかった。同様に、別のトランスポゾン誘発変異は、ＵＤＰ-グルコース-４・エピメラーゼ活性を除去する。

【００２４】ＬＰＳは、最初にリピドＡ部分が合成された後、内側コアおよび外側コアの糖残基が続けて付加されることにより生合成される。コア(例えば、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の$GlcNAc\text{-}Hep_2(PEA)\text{-}KDO_2$-リピ

ドA)に対するヘキソース単位(例えば、グルコースおよびガラクトースなど)の付加が、この生合成に関与する一連の酵素により触媒されることが知られている。例えば、グルコースは、グルコキナーゼによりグルコース-6-ホスフェートに変換される。ホスホグルコムターゼ酵素は、グルコース-6-ホスフェートをグルコース-1-ホスフェートに変換する。次いで、グルコース-1-ホスフェートは、UTP-グルコース-1-ホスフェート-ウルジルトランスフェラーゼによりUDP-グルコースに変換される。また、UDP-グルコースは、UDP-グルコース-4-エピメラーゼによりUDP-ガラクトースに変換される。次いで、これらのUDP中間体由来のヘキソース単位は、トラスフェラーゼにより触媒されたLPSのコアに転移する。これらの転移活性の喪失や、UDP-グルコースおよびUDP-ガラクトース生成の原因となる酵素により、内側コアのみを含有するLPS構造が調製される。コアLPSを調製するのに必要な経路は、ホスホグルコムターゼ、UTP-グルコース-1-ホスフェート-ウルジルトランスフェラーゼ、UDP-グルコース-4・エピメラーゼ、あるいは、UDP-グルコースまたはUDPガラクトース・トランスフェラーゼの喪失により影響を受けない。それゆえ、これらの酵素に欠失があると、LPSの鎖停止となる。

【0025】所定のグラム陰性菌の如何なる形態のLPSであっても、本発明の接合体を調製するのに利用しうるので、本発明の保存LPS部分は、接合前に脱O-アシル化されていることが好ましい。LPS構造は、例えば、ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),250:1926-1932(1975)およびジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),256:7305-7310(1981)に記載されているような、水酸化ナトリウムを用いた、その穏やかなアルカリ加水分解により、あるいは、例えば、ヨーロピアン・ジャーナル・オブ・バイオケミストリー(Eur. J. Biochem.),177:483-492(1988)に記載されているような、穏やかなヒドラジン処理により、都合よく脱O-アシル化することができる。LPSを脱O-アシル化することは、その免疫原性を向上させ、かつ、その毒性を減少させることが見い出されている。あるいは、非毒性LPS部分は、例えば、WO97/19688に記載の方法により、病原性グラム陰性菌の非毒性変異体から単離することができる。

【0026】本発明の抗原接合体を製造するには、保存LPS部分を適当なリンカー化合物を介して担体タンパクに結合させる。かかるリンカー化合物の使用は、当該分野で公知であり、例えば、コンジュゲート・ワクチンズ(Conjugate Vaccines),クルイーゼ(Cruise)ら、48-114, カルガー・パブリッシング(Karger Publishing)(1989)に考察されている。

【0027】例えば、保存LPS構造のリピドA部分または内側コア部分上の反応基は、公知の異種二官能性および同種二官能性の結合剤に結合させることができる。異種二官能性の結合剤は、異種反応性末端を含有し、保存LPSを有する中間体を生成する。これらの中間体は、一方の末端でLPSに結合し、反対側の末端が反応性基を含有する。同種二官能性の結合剤も、2つの反応性基を含有するが、それらは同じである。一般に、結合剤は、アミノ基、ヒドロキシル基またはカルボキシル基を介して、保存LPS部分を担体タンパクに結合させる。アミノ基およびヒドロキシル基による結合は、保存LPSのリピドAまたは内側コアの糖と形成される。カルボキシル基による結合は、内側コアのKDOと形成される。結合剤の反対側の末端は、さらに担体タンパクと反応するためのスルフヒドリル基を含有することが好ましい。

【0028】本発明で用いるのに適した結合剤としては、例えば、スルホスクシンイミジル-6-(3-[2-ピリジルジチオ]プロピオンアミド)-ヘキサノエート(スルホ-LC-SPDP)、スクシンイミジル-6-(3-[2-ピリジルジチオ]プロピオンアミド)-ヘキサノエート(LC-SPDP)、トラウト(Traut)試薬(2-イミノチオラン)、N-スクシンイミル-S-アセチルチオアセテート(SATA)、N-スクシンイミジル-3-(2-ピリジルジチオ)プロピオネート(SPDP)、スクシンイミジルアセチルチオプロピオネート(SATP)、スクシンイミジル-4-(N-マレイミドメチル)シクロヘキサン-1-カルボキシレート(SMCC)、マレイミドベンゾイル-N-ヒドロキシスクシンイミドエステル(MBS)、N-スクシンイミジル(4-ヨードアセチル)アミノベンゾエート(SIAB)、スクシンイミジル4-(p-マレイミドフェニル)ブチレート(SMPB)、ブロモ酢酸-N-ヒドロキシスクシンイミド(BANS)エステル、1-エチル-3-(3-ジメチルアミノプロピル)カルボジイミド(EDAC)、アジピン酸ジヒドラジド(ADH)、システタミンおよびジチオビス(スクシンイミジルプロピオネート)(DTSSP)などが挙げられる。

【0029】LPSの保存部分は、非アミノ含有緩衝溶液(例えば、リン酸緩衝食塩水または重炭酸ナトリウム水溶液)中、中性から弱アルカリ性のpHで、適当な時間(例えば、室温で約1時間)にわたって、結合剤と反応させる。次いで、中間体を、適当な手段(例えば、ゲル沪過)により、未反応の反応物から取り出す。次いで、担体タンパクは、上記の群から選択される適当な結合剤との反応により活性化される。次いで、保存LPS中間体は、適当な条件下で活性化担体タンパクと反応させて、本発明の接合体を得る。

【0030】スルフヒドリル基による結合の場合には、LPS-結合剤中間体を適当な還元剤またはヒドロキシアミンと反応させ、結合剤上のジスルフィド結合を還元して、遊離のスルフヒドリル基を露出させる。適当な還元剤としては、ジチオスレイトール(DTT)およびメル

カプトエタノールが挙げられる。次いで、スルフヒドリルの露出した中間体を活性化担体タンパクと反応させて、チオエーテルで結合した共有結合型の接合体を得る。



*【0031】スルフヒドリル基による抗原接合体の形成を以下に概略的に示す。

【0032】

【化1】

(A)

$LPS\text{-}NH_2 + NHS\text{-}CO\text{-}(CH_2)_5\text{-}NH\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}ピリジル \rightarrow$

(LC-SPDP)

$LPS\text{-}NH\text{-}CO\text{-}(CH_2)_5\text{-}NH\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}ピリジル \xrightarrow{還元剤}$

$LPS\text{-}NH\text{-}CO\text{-}(CH_2)_5\text{-}NH\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}SH$

(B)

$タンパク\text{-}NH_2 + NHS\text{-}CO\text{-}CH_2\text{-}Br \rightarrow$

(ブロモ酢酸N-ヒドロキシスクシンイミド) エステル

$タンパク\text{-}NH\text{-}CO\text{-}CH_2Br$

(C)

(A) + (B) ⟶

$LPS\text{-}NH\text{-}CO\text{-}(CH_2)_5\text{-}NH\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CO\text{-}NH\text{-}タンパク$

(タンパク/LPS接合体)

【0033】あるいは、例えば、モリソン，アール・ティー(Morrison, R.T.)およびボイド，アール・エヌ(Boyd, R.N.)、オーガニック・ケミストリー(Organic Chemistry),アリン・アンド・ベイコン，インク(Allyn and Bacon, Inc.),875-905(1966)に開示されているように、糖構造上のビシナル・ヒドロキシル基の過ヨウ素酸酸化により、あるいは、例えば、アヴィゲルド(Avigaeld)ら、ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.),237:2736(1962)に開示されているように、非還元末端ガラクトースまたはN-アセチルガラクトサミン残基を含有するＬＰＳをガラクトース・オキシダーゼで処理して、６位の炭素上におけるヒドロキシル基をアルデヒド基に変換することにより、アルデヒドをＬＰＳ構造上に露出させてもよい。次いで、アルデヒドを含有するＬＰＳは、例えば、還元アミノ化により、適当な担体タンパクに結合させることができる。

【0034】さらに別の方法では、本発明の接合体は、※1-エチル-3-(ジメチルアミノプロピル)ジカルボジイミド塩酸塩(ＥＤＡＣ)などのカルボジイミド試薬を用いて、ＬＰＳ上のカルボキシル基を介してＬＰＳの保存部分と担体タンパクとを結合させることにより、形成してもよい。この方法では、ＬＰＳ糖のカルボキシル基を担体タンパクのカルボキシル基に結合させることにより、これらの接合体を調製することが好ましい。この場合は、まず、保存ＬＰＳをＥＤＡＣの存在下でアジピン酸ジヒドラジド(ＡＤＨ)などの適当なリンカー化合物と反応させる。次いで、担体タンパクをＥＤＡＣなどの適当なリンカー化合物の存在下でＡＤＨ-ＬＰＳ中間体と反応させればよい。カルボキシル基で結合した接合体が得られる。

【0035】カルボキシル基の結合による抗原接合体の形成を以下に概略的に示す。

【0036】

【化2】

(A)

$LPS\text{-}COOH + NH_2NH\text{-}CO\text{-}(CH_2)_4\text{-}CO\text{-}NH\text{-}NH_2 \xrightarrow{EDAC/NHS}$

(ADH)

$LPS\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}(CH_2)_4\text{-}CO\text{-}NH\text{-}NH_2$

(B)

$(A) + タンパク\text{-}COOH \xrightarrow{EDAC/NHS}$

$LPS\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}(CH_2)_4\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}タンパク$

(カルボキシル結合-ＡＤＨ接合体)

NHS＝N-ヒドロキシスクシンイミド

【0037】さらに別の方法では、本発明の接合体は、★50★ＬＰＳ糖のカルボキシル基を担体タンパクのアミノ基に

結合させることにより、形成してもよい。この場合は、まず、保存ＬＰＳをＥＤＡＣの存在下でシスタミンと反応させる。次いで、シスタミン-ＬＰＳ中間体のスルフヒドリル基をジチオスレイトールなどの還元剤で露出させ、最終的にブロモ-アセチル化担体タンパクと反応させる。

【００３８】本発明の抗原接合体の調製に有用な担体タンパクとしては、細菌毒素およびトキソイド(例えば、破傷風毒素またはトキソイド、ジフテリア毒素またはトキソイド、ジフテリア非毒性変異体毒素ＣＲＭ$_{197}$(例えば、イムノケミストリー(Immunochem.),9:891-906(1972)に記載)、シュードモナス属菌(Pseudomonas)の外毒素Ａ、コレラ毒素またはトキソイド、Ａ型レンサ球菌毒素、ストレプトコッカス・ニュモニエ(Streptococcus pneumoniae；肺炎レンサ球菌)の肺炎レンサ球菌溶血毒素など)；ボルデテラ・ペルツッシス(Bordetella pertussis；百日咳菌)のフィラメント状赤血球凝集素(ＦＨＡ)またはＦＨＡ断片；ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)の線毛またはピリン；ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の線毛またはピリン；細菌の外膜タンパク(例えば、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の外膜タンパク複合体(例えば、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のクラス１外膜タンパクおよびクラス３外膜タンパク)；ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)の外膜タンパク)；ストレプトコッカス属菌(Streptococcus；レンサ球菌)のＣ５Ａペプチダーゼ；およびモラクセラ・カタラーリス(Moraxella catarrhalis)の偏在表面タンパクが挙げられる。好ましい担体タンパクは、ＣＲＭ$_{197}$である。

【００３９】本発明の抗原接合体を含有するワクチンは、ワクチン抗原に対する免疫応答を増大させることが知られている様々なアジュバントを含有することが有利な場合もある。かかるアジュバントは、患者の免疫系の非特異的な刺激により、抗体応答を増大させると考えられる。アジュバントの使用は、当該分野で公知であり、例えば、「ワクチン・デザイン(Vaccine Design)：ザ・サブユニット・アンド・アジュバント・アプローチ(The Subunit and Adjuvant Approach)」、パウエル(Powell)ら、プレナム・プレス(Plenum Press)(1995)に記載されている。本発明の接合体を含有するワクチンに用いるのに適当なアジュバントの例としては、リン酸アルミニウム、水酸化アルミニウム、モノホスホリルリピドＡ、３-脱アシル化モノホスホリルリピドＡ、ＱＳ-２１(ジャーナル・オブ・イムノロジー(J. Immunol.),146:431-437(1991)に開示)だけでなく、アルミニウム化合物と組み合わせた様々な界面活性剤(例えば、トリトン(Triton$^{TM}$)Ｘ１００、ツウィッタージェント(zwittergent)およびデオキシコレート)が挙げられる。一般に、本発明の接合体に対する抗体応答は、ワクチンに１種またはそれ以上のアジュバントを含有させることにより、実質的に増大される。

【００４０】ワクチン製剤を必要とする個体に投与するのに適した多くの方法が知られている。適当な投与方法としては、皮内、筋肉内、腹腔内、静脈内、動脈内、膣内、皮下、眼内、鼻腔内および経口投与が挙げられる。

【００４１】本発明の抗原接合体を含有するワクチン製剤は、例えば、等張性溶液、食塩水、リン酸緩衝食塩水などの生理学的に許容される担体中における接合体からなる。ワクチン製剤は、個体に予防上有効量で投与される。

【００４２】数多くの様々な異なる菌種に対する交差反応性により、本発明の抗原接合体は、ＬＰＳ産生菌により引き起こされる疾患に対する哺乳動物の免疫反応を生じさせるワクチンの成分として効果的である。本発明の接合体を含有するワクチンは、当業者に公知の方法および材料を用いて調製すればよい。

【００４３】本発明の接合体により生じる抗体を用いて、感染した個体の血液試料、体液または生検試料を試験することにより、感染がＬＰＳ産生菌により引き起こされたか否かを調べうる。治療および予防用途には、本発明のワクチンを用いることだけでなく、それを用いて得られた抗体の使用も包まれる。本発明の抗原接合体を用いた活性免疫は、細菌感染または疾患の予防に有用でありうる。

【００４４】本発明のワクチンは、様々グラム陰性菌(例えば、サルモネラ属菌(Salmonella)、エシェリキア・コリ(Escherichia coli；大腸菌)、ナイセリア属菌(Neisseria)、ヘモフィルス属菌(Haemophilus)、シゲラ属菌(Shigella；赤痢菌)、クレブシエラ属菌(Klebsiella)およびシュードモナス属菌(Pseudomonas))により引き起こされる敗血症ショックの予防に有用でもある。場合によっては、従来の抗生物質で治療した後の細菌の死滅細胞から有意な量のＬＰＳが放出されることが発見されている[ジャーナル・オブ・インフェクシャス・ディジーズ(J. Infect. Dis.),157:567-568(1988)]。これは、これらの患者に外毒素誘発性の合併症をもたらしうる。

【００４５】敗血症ショックおよびその関連合併症を防止するあるアプローチは、関与しているかもしれない細菌のＬＰＳの共通コア領域に対するモノクローナル抗体またはポリクローナル抗体を患者に投与することである。かかる抗血清は、細菌により過剰に産生されたＬＰＳの毒性効果を防止しうる。エシェリキア・コリ(Escherichia coli；大腸菌)またはサルモネラ・ミネソタ(Salmonella minnesota)免疫原の変異体により産生されるＬＰＳを用いた、動物モデルにおけるＬＰＳ抗体治療の可能性は、アップルメルク(Applemelk)およびコーエン(Cohen)により、「バクテリアル・エンドトキシック・リポポリサッカライズ(Bacterial Endotoxic Lipopolysacch

arides；細菌外毒素リポ多糖)-第II巻」、イムノファーマコロジー・アンド・パソロジー(Immunopharmacology and Pathology)，シー・アール・シー・プレス(CRC Press)，(1992)に概説されている。

【００４６】以下の実施例により、本発明をさらに詳しく説明するが、これらの実施例は本発明を限定することを意図したものではない。

【００４７】

【実施例】細菌および増殖条件の選択：ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)ＮＭＢ-Ｒ６株のＴｎ９１６誘発ＬＰＳ変異体を、ジョウ(Zhou)ら、ジャーナル・オブ・バイオロジカル・ケミストリー(J. Biol. Chem.)，269:11162-11169(1994)に記載の方法により構築した。この株は、約３．１～３．２ＫＤａの分子量を有するＬＰＳを発現する表現形の安定な変異体である。この変異株がグルコース-6-ホスフェートをグルコース-1-ホスフェートに変換できないことから、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の保存コアＬＰＳ構造のみを含有する切断ＬＰＳ部分が得られることが示されている。この変異株により産生されるＬＰＳの構造は、ＧｌｃＮＡｃ-$Hep_2$ホスホエタノールアミン-$KDO_2$-リピドAとして同定されている。

【００４８】この変異株を、ＧＣ寒天プレート培地上、３５℃、５％$CO_2$中で６時間増殖させた。次いで、この固形寒天培養物の細胞を、０．２％酵母エキス透析物を含有する補足液体培地中で１８時間増殖させた。次いで、この培養物をフェルンバッハ(Fernbach)フラスコに移し、さらに１８～２４時間増殖させた。次いで、これらの細胞を熱的に死滅させ、ＬＰＳ構造を精製するために遠心分離で採取した。

【００４９】ＬＰＳ精製：上記のＮＭＢ-Ｒ６細胞から、ウー(Wu)ら、アナリティカル・ケミストリー(Anal. Chem.)，160:298-289(1987)に記載の熱フェノール-水抽出法により、ＬＰＳを抽出し、超遠心分離により精製した。より具体的には、細胞ペレットを３倍容量(３ｍＬ緩衝液/ｇ湿重量)のリン酸緩衝液(ｐＨ７．１)(５ｍＭＥＤＴＡおよび０．０２％アジ化ナトリウムを含有)に懸濁させた。次いで、この懸濁液に、濃度２ｍｇ/ｍＬのリソチーム(シグマ・ケミカル・カンパニー(Sigma Chemical Co.)から入手可能)を加えた。次いで、この混合物を４℃で一晩消化させた。この懸濁液を３７℃にし、さらに、濃度１００μｇ/ｍＬのＲＮＡｓｅおよびＤＮＡｓｅで３時間消化させた。次いで、この消化物を７０℃にし、これに７０℃の等量のフェノールを加えた。この混合物を１５分間抽出し、次いで、この懸濁液を４℃に冷却し、１０，０００ｇで３０分間遠心分離した。水相を回収し、フェノール相を７０℃の等量の水で１５分間再抽出した。次いで、この相を１０，０００ｇで３０分間遠心分離し、次いで、水相を分離した。合わせた上澄水溶液に酢酸ナトリウムを濃度５ｍｇ/ｍＬで加えた。次いで、この混合物に２倍容量の氷冷アセトンを加え、ＬＰＳを４℃で一晩かけて沈殿させた。１０，０００ｇで３０分間遠心分離することにより、沈殿したＬＰＳを分離した。回収したＬＰＳを滅菌水に懸濁し、１０５，０００ｇで３回、３時間超遠心分離した。最終ペレットを少量の滅菌水に懸濁し、次の実験に用いた。典型的には、１ｇ湿重量のＮＭＢ-Ｒ６細胞から３ｍｇのＬＰＳを精製した。

【００５０】担体タンパクＣＲＭ$_{197}$への接合

次いで、上記のように精製したＬＰＳを、８０℃で４５ｍＭのＮａＯＨと２０分間反応させることにより、脱Ｏ-アシル化した。次いで、この脱Ｏ-アシル化物をＨＣｌで中和し、０．１Ｍ $NaHCO_3$を溶離液として用いるバイオゲル(Biogel)Ｐ６カラム上のゲル沪過で精製した。次いで、脱Ｏ-アシル化ＬＰＳ(以下、「ＤｅＡ-ＬＰＳ」と呼ぶ)を、下記の方法を利用して保存ＬＰＳ構造上の糖のアミノ基を担体タンパクのアミノ基に結合させることにより、担体タンパクＣＲＭ$_{197}$に接合させる。ＣＲＭ$_{197}$は、ジフテリア毒素の非毒性変異体タンパクであり、ヒト用の糖接合体ワクチンを商業的に生産するための担体タンパクとして用いられている。

【００５１】長鎖スルホ-Ｎ-スクシンイミジル-３-(２-ピリジルジチオ)-プロピオネート(ピアス・ケミカル・カンパニー(Pierce Chemical Company)から入手可能なスルホＬＣ-ＳＰＤＰ)を用いて、ＤｅＡ-ＬＰＳの第一級アミノ基をチオレート化した。スルホＬＣ-ＳＰＤＰを、１：１(w/w)の割合で、０．１Ｍ $NaHCO_3$(ｐＨ７．９)中における１５ｍｇのＬＰＳに加えた。次いで、この混合物を室温で１時間インキュベートした。反応の終点で、この混合物を、０．１Ｍ $NaHCO_3$で平衡化したバイオゲル(Biogel)Ｐ６カラムで精製した。回収した画分を、ケレチ(Keleti)およびレデラー(Lederer)、バイオケミストリー・アンド・バイオフィジクス(Biochem. Biophys.)，74:443-450(1974)に記載の方法により、ＫＤＯについてアッセイし、ＫＤＯを含有する画分をプールした。ＬＰＳのＳＰＤＰ誘導体中に存在するＮ-ピリジルジスルフィドは、５０～１００ｍＭ ジチオスレイトール(ＤＴＴ)で還元し、上記のようにバイオゲル(Biogel)Ｐ６カラム上でゲル沪過した。チオール化物を含有するＫＤＯ陽性の画分を再びプールした。ＤｅＡ-ＬＰＳのオリゴ糖のチオール化は、エルマン，ジー・エル(Ellman, G.L.)，アーカイブズ・オブ・バイオケミストリー・アンド・バイオフィジックス(Arch. Biochem. Biophys.)，74:443-450(1958)に記載の反応に従ってモニターした。０．１ｍＬの上記物質を０．１ｍＬのエルマン試薬(すなわち、１０ｍＬのリン酸緩衝食塩水(ｐＨ８．０)中における４０ｍｇの５，５'-ジチオビス(２-ニトロ安息香酸))と混合した。１５分間インキュベートした後、吸光度を４１２ｎｍで測定した。システインを標準

的なスルフヒドリル試薬として用いた。

【００５２】$CRM_{197}$担体タンパクを、ベルナトビッツ(Bernatowitz)およびマツエダ(Matsueda)、アナーレン・デア・バイオヘェミー(Anal. Biochem.),155:95-102(1986)に記載の方法により、ブロモアセチル化した。１００mg/mLのジメチルホルムアミド中におけるブロモ酢酸-N-ヒドロキシスクシンイミドエステル(シグマ・ケミカル・カンパニー(Sigma Chemical Co.)から入手可能)を、4℃において、１：１(w/w)の割合で、３mLの上記タンパク(0.1M $NaHCO_3$中)に滴下した。この溶液を混合し、室温で1時間インキュベートした。次いで、この反応混合物を、上記のように、バイオゲル(Biogel)Ｐ６カラム上でゲル沪過し、ブロモアセチル化タンパクを含有するボイド画分をプールした。担体タンパク上のアミノ基のブロモアセチル基への誘導体化は、遊離アミノ基の量の減少によりモニターした。

【００５３】次いで、0.1M $NaHCO_3$中におけるブロモアセチル化$CRM_{197}$を、１：1.5(w/w)の割合(タンパク/ＬＰＳ)で、0.1M $NaHCO_3$中におけるチオール化ＤｅA-ＬＰＳに加えた。この反応混合物を4℃で一晩インキュベートした。最終的な接合体(以下、「ＤｅA-ＬＰＳ-ＳＰＤＰ-CRM」と呼ぶ)を、0.1M $NaHCO_3$/1mM ＥＤＴＡ(ｐＨ7～9)で平衡化したバイオゲル(Biogel)Ｐ３０(バイオ-ラッド(Bio-Rad))カラム上のゲル沪過で精製した。

【００５４】免疫原性の測定：上で調製したＤｅA-ＬＰＳ-ＳＰＤＰ-CRM接合体の免疫原性を、下記の方法により、スイス・ウェブスター(Swiss Webster)マウスで測定した。6～8週齢の雌マウスのグループ(１０匹/グループ)を、１０μｇ ＬＰＳ、１０μｇ ＤｅA-ＬＰＳ、１０μｇ ＤｅA-ＬＰＳ-ＳＰＤＰ(すなわち、非接合型中間体)および１０μｇのＤｅA-ＬＰＳ-ＳＰＤＰ-CRM接合体で皮下免疫した。また、対照として供するマウスに、１０μｇ $CRM_{197}$を投与した。これらの免疫原の各々は、さらに、アジュバントとして、２０μｇのＱＳ-２１(アクエラ(Aquila)から入手可能)を、投与量あたりリン酸緩衝食塩水(ＰＢＳ)を含有する０.１mLの最終容量で含有していた。さらに別の１つのグループを、ＱＳ-２１アジュバントを用いることなく、１０μｇのＬＰＳで免疫した。これらの動物は、第0週、第３週および第６週に免疫したが、各免疫前に抗体測定用の血液試料を採取した。抗体測定用の血液試料は、さらに第８週にも採取した。

【００５５】ＬＰＳ抗体レベルは、下記に示すナイセリア・メニンジティディス(Neisseriameningitidis；髄膜炎菌)のＲ６株および他の野生型株ならびに免疫型特異的な株由来の精製ＬＰＳに対する酵素結合抗体免疫吸着アッセイ(ＥＬＩＳＡ)法で測定した。免疫型特異的な株は、ワシントン州のウォルター・リード・アーミィ・メディカル・センター(Walter Reed Army Medical Center)から得た。ＬＰＳは、上記の熱フェノール-水抽出法により、これらの株から精製した。

【００５６】精製したＬＰＳを、内毒素を含まないＰＢＳに下記の濃度で希釈した。ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)Ａ１株、Ｈ４４/７６株、２９９６株および免疫型Ｌ１、Ｌ２、Ｌ３、Ｌ４、Ｌ５、Ｌ６、Ｌ７、Ｌ８、Ｌ１０、Ｌ１１およびＬ１２については、１０μｇ/mＬ；ならびに、Ｒ６株については、２.５μｇ/mＬ。ポリスチレン製マイクロタイタープレートを、各ウェルあたり１００μＬの希釈したＬＰＳ含有混合物で被覆し、３７℃で3時間インキュベートした後、4℃で一晩保存した。次いで、自動プレート洗浄機を利用して吸引することにより、これらのプレートから非結合ＬＰＳを除去した。次いで、これらのプレートに各ウェルあたり１５０μＬのＰＢＳ/０.１％ゼラチンを加えた後、これらのプレートを３７℃で６０分間インキュベートした。このインキュベーションの後、および、すべてのその後の工程の間、自動プレート洗浄機を用いて、これらのプレートをＰＢＳおよび０.１％ツイーン(Tween)２０の混合物で洗浄した。

【００５７】試験マウス血清を、ＰＢＳ、０.０５％ツイーン(Tween)２０および０.１％ゼラチンの混合物中に連続希釈した。各ウェルあたり１００μＬの希釈液を上記プレートに加えた。これらのプレートを３７℃で６０分間インキュベートした。次いで、ＰＢＳおよび０.５％ツイーン(Tween)２０の混合物中に希釈したヤギ抗マウスＩｇGアルカリホスファターゼ(サザン・バイオテクノロジー(Southern Biotechnology)製)を、各ウェルあたり１００μＬの量で加え、３７℃で６０分間インキュベートした。ジエタノールアミン緩衝液中におけるｐ-ニトロフェノールホスフェートの１mg/mＬ溶液１００μＬを用いて、発色させた。これらの材料を室温で６０分間反応させた後、各ウェルあたり５０μＬの３ＮＮａＯＨを加えることにより、この反応を停止させた。４０５nm試験フィルターおよび６９０nm参照フィルターを備えた自動ＥＬＩＳＡ読取り装置を用いて、吸光度の値を求めた。

【００５８】Ｒ６株由来の同種ＬＰＳに対するＤｅA-ＬＰＳ-ＳＰＤＰ-CRM接合体の免疫原性を立証する第０週、第３週、第６週および第８週のデータを表１に示す。このデータから明らかなように、接合体は有意な追加刺激可能なＩｇG抗体応答を生じさせた。

【００５９】

【表１】

21　　22

| 免疫原 | R6 LPSに対するIgG抗体応答* | | | |
|---|---|---|---|---|
| | 第0週 | 第3週 | 第6週 | 第8週 |
| LPS | <50 | 365 | 769 | 17,633 |
| LPS + QS-21 | <50 | 1,079 | 24,225 | 84,491 |
| DeA-LPS + QS-21 | ND | ND | ND | 221 |
| DeA-LPS-SPDP-$CRM_{197}$ + QS-21 | <50 | 203 | 6,647 | 56,062 |
| $CRM_{197}$ + QS-21 | <50 | <50 | <50 | <50 |

DeA-LPS:　脱O-アシル化ＬＰＳ，非接合
DeA-LPS-SPDP:　活性化された脱O-アシル化ＬＰＳ，非接合
DeA-LPS-SPDP-CRM:　ＳＰＤＰにより$CRM_{197}$に接合した脱O-アシル化ＬＰＳ

ND＝実施せず

*これらの値は、希釈した血清O.D.値0.1を与える終末点の希釈率を表す。

【0060】また、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の様々な株の異種ＬＰＳに対する本実施例で製造した接合体の交差反応免疫原性を下記の方法により調べた。得られたデータを表2に示す。調べた株は、Ａ1、Ｒ6、Ｈ44/76、29　20　96、免疫型Ｌ1、Ｌ2、Ｌ3、Ｌ4、Ｌ5、Ｌ6、Ｌ*７、Ｌ8、Ｌ10、Ｌ11およびＬ12であった。表2から明らかなように、ＬＰＳ-タンパク接合体は、特に非接合型ＬＰＳに比べて、有意な抗体応答を生じさせた。

【0061】

【表2】

| 株 | DeA-LPS-SPDP | DeA-LPS-SPDP-$CRM_{197}$ |
|---|---|---|
| R6 | 443 | 33,067 |
| A1 | <100 | 36,923 |
| H44/76 | 147 | 24,811 |
| 2996 | <100 | 11,467 |
| L1 | <100 | 11,963 |
| L2 | 350 | 4500 |
| L3 | 304 | 7,936 |
| L4 | 412 | 12,820 |
| L5 | 259 | 16,251 |
| L6 | 129 | 11,600 |
| L7 | <100 | 16,533 |
| L8 | 232 | 16,278 |
| L10 | 141 | 11,982 |
| L11 | <100 | 3200 |
| L12 | 142 | 4,870 |

DeA-LPS-SPDP:　活性化された脱O-アシル化ＬＰＳ，非接合
DeA-LPS-SPDP-CRM:　ＳＰＤＰによりＣＲＭ...に接合した脱O-アシル化ＬＰＳ

【0062】さらに、数種のグラム陰性菌の様々な株由来の精製ＬＰＳに対するウェスタンブロット分析により、抗ＬＰＳ接合体抗血清(ＤｅＡ-ＬＰＳ-ＳＰＤＰ-ＣＲＭに対する抗血清)の交差反応性を調べた。まず、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)、型別不能ヘモフィルス・インフルエンゼ(non-typeable Homophiles influenzae；インフルエンザ菌)、ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)、モラクセラ・カタラーリス(Moraxella catarrhalis)およびヘリコバクター・ピロリ(Helicobacter p※50※ylori)の精製ＬＰＳ試料をプロテアーゼで消化し、標準的なＳＤＳ-ＰＡＧＥ(１８%)分離法に供した。次いで、標準的なウェスタンブロット法により、これらの試料をニトロセルロースメンブレンに転写した。このメンブレンを、ＰＢＳ/0.05%ツイーン(Tween)２０の混合物中における３%ウシ血清アルブミン(ＢＳＡ)で３０分間ブロックし、試験マウス血清の１：１００希釈液と反応させた。次いで、これらのブロットをＰＢＳ/0.05%ツイーン(Tween)２０の混合物で洗浄し、ＰＢＳ/0.05%ツイーン(Tween)２０の混合物で希釈したヤギ

抗マウスＩｇアルカリホスファターゼと共にインキュベートした。洗浄した後、5-ブロモ-4-クロロ-3-インドリルホスフェート(ＢＣＩＰ)/ニトロブルーテトラゾリウム濃縮物(ＮＢＴ)ホスファターゼ基質系を製造業者(キルケガード・アンド・ペリー・ラボラトリーズ，インク(Kirkegaard and Perry Laboratories, Inc.)、メリーランド州)の記載どおりに用いて、これらのブロットを展開させた。展開法は、ガラス容器中でＢＣＩＰおよびＮＢＴ濃縮物の各々１部を１０部のトリス(Tris)緩衝液と混合し、これらの混合物をブロットに加えることからなっていた。発色後、ブロットを試薬級の水ですすぐことにより、反応を停止させた。

【００６３】ウェスタンブロット分析の結果を図８に示す。この図において、レーン１は低分子量マーカー(BRL lows)、レーン２はブランク試料、レーン３はナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)Ａ１株、レーン４および５は型別不能ヘモフィルス・インフルエンゼ(non-typeable Haemophilus influenzae；型別不能インフルエンザ菌)ＴＮ１０６株およびＰ８６０２９５株、レーン６および７はナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)５２４０７Ｐ＋株および１７５６Ｐ＋株、レーン８～１０はヘリコバクター・ピロリ(Helicobacter pylori)１１０３株、４３５７９株および１１０５株、レーン１１～１３はモラクセラ・カタラーリス(Moraxella catarrhalis)１２３０:３５９株、２５２３８株および４３０：３４５株、レーン１４および１５はナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)ＬＢ２株およびＰｇｈ３-１株である。

【００６４】図８から明らかなように、ヘリコバクター・ピロリ(Helicobacter pylori)を除いて、各微生物の＊ＬＰＳは、抗血清と強く反応した。強度は小さいが、ヘリコバクター・ピロリ(Helicobacter pylori)由来のＬＰＳに対しても、わずかな交差反応性が存在したようである。これらの結果は、明らかに、ナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)のＬＰＳ接合体から生じた抗体が数多くの他のグラム陰性菌と交差反応したことを示している。

【００６５】さらに、Ｒ６株、Ａ型株(Ａ１)、ならびに、２つのＢ型株Ｈ４４/７６および２９９６を用いて、抗血清の殺菌活性を調べた。血清試料を５μＬのＰＣＭ(ＣａおよびＭｇを含有するＰＢＳ)に希釈し、この希釈液を、２～５×$10^3$のナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)(１０μＬ)、ヒト血清補体(１０μＬ)およびＰＣＭ(２５μＬ)を含有する反応混合物に加えた。次いで、この混合物を５％$CO_2$中、３６℃で４５分間インキュベートした。次いで、２００μＬのＰＢＳで希釈することにより、この反応を停止させた。次いで、この混合物の２つの既知量(５０μＬ)をＧＣ寒天プレート上にプレートし、３６℃、５％$CO_2$中で、さらにインキュベートした。次いで、殺菌力価($BC_{50}$)を求めた。殺菌力価は、このアッセイにおいて、コロニーを形成するナイセリア・メニンジティディス(Neisseria meningitidis；髄膜炎菌)の５０％を死滅させる抗血清の希釈率の逆数を表す。データを下記の表３に示す。

【００６６】表３から明らかなように、接合体抗血清は、様々なＬＰＳ免疫型を表現する細菌を死滅させることができた。かかる接合体は、追加刺激可能なＴ細胞依存性ＩｇＧ応答を誘発した。

【００６７】

【表３】

$BC_{50}$力価

| 免疫原 | H44／76株 | 2996株 | A1株 | R6株* |
|---|---|---|---|---|
| LPS | <50 | <50 | <50 | <50 |
| DeA-LPS-SPDP | <50 | <50 | <50 | ND |
| DeA-LPS-SPDP-$CRM_{197}$ | 50 | 70 | 350 | <50 |
| 正常マウス血清 | <50 | <50 | <50 | <50 |

*陽性対照抗血清(マウス抗Ａ１ＬＰＳ)をアッセイに用いて、力価１００を与えた。

ND=実施せず

DeA-LPS-SPDP:　活性化された脱O-アシル化ＬＰＳ，非接合
DeA-LPS-SPDP-CRM:　ＳＰＤＰにより$CRM_{197}$に接合した脱O-アシル化ＬＰＳ

【００６８】したがって、上記のデータから容易にわかるように、本発明の抗原接合体は、所定の細菌のＬＰＳに対する有意の免疫応答を生じさせる。さらに、このデータは、本発明の接合体が、細菌の様々な株に対してだけでなく、細菌の様々な種に対しても、交差反応応答を誘発することを示している。

【００６９】本発明は、その精神および本質的な属性から※逸脱することなく、他の特定の形態で具体化してもよく、したがって、本発明の範囲を示していることから、上記の発明の詳細な説明よりむしろ、特許請求の範囲を参照すべきである。

【００７０】

【発明の効果】本発明によれば、グラム陰性菌の所定の種に対する免疫原性応答だけでなく、所定の属に属する

グラム陰性菌の異なる株または血清型や、好ましくは異なる属のグラム陰性菌に対する交差反応免疫応答を誘発する抗原接合体およびそれを含有するワクチンが提供される。

【図面の簡単な説明】

【図1】 サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)のリピドA-内側コアの典型的な構造を示す。

【図2】 サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)のO抗原または反復多糖の典型的な構造を示す。

【図3】 サルモネラ・チフィムリウム(Salmonella typhimurium；ネズミチフス菌)のヘプタアシルリピドAの構造を示す。

【図4】 ナイセリア属菌(Neisseria)のコアLOS構造を示す。

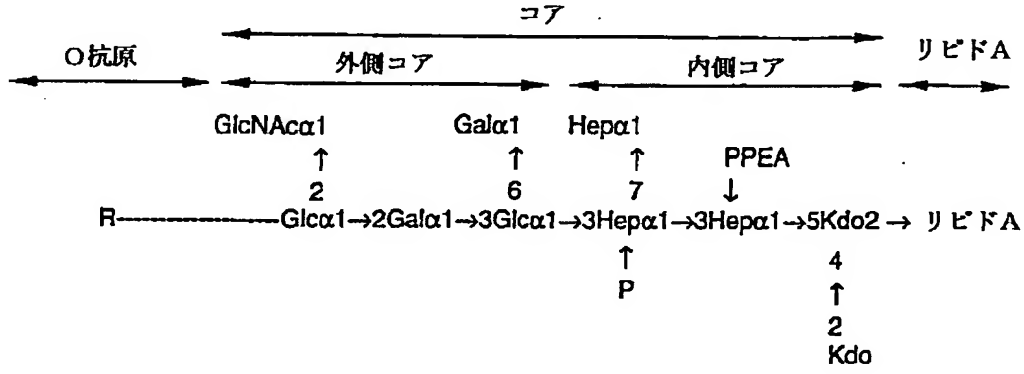
【図5】 ヘモフィルス属菌(Haemophilus)のコアLOS構造を示す。

【図6】 ヘモフィルス・インフルエンゼ(Haemophilus influenzae；インフルエンザ菌)2019株のコアLOS構造を示す。

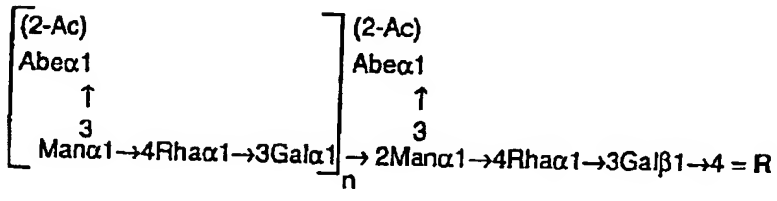
【図7】 ナイセリア・ゴノレエ(Neisseria gonorrhoeae；淋菌)1291株のコアLOS構造を示す。

【図8】 様々な細菌由来のLOSに対する抗LOS-接合体抗血清のウェスタンブロット分析の結果を示す。

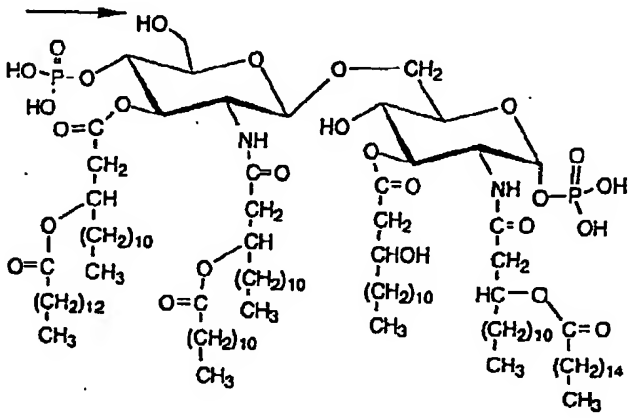
【図1】



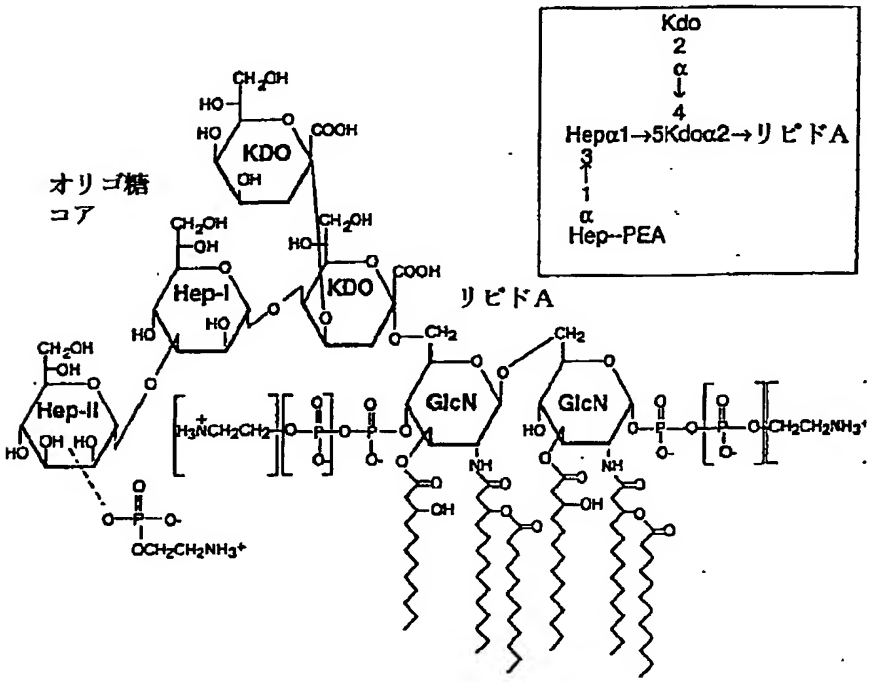

【図2】



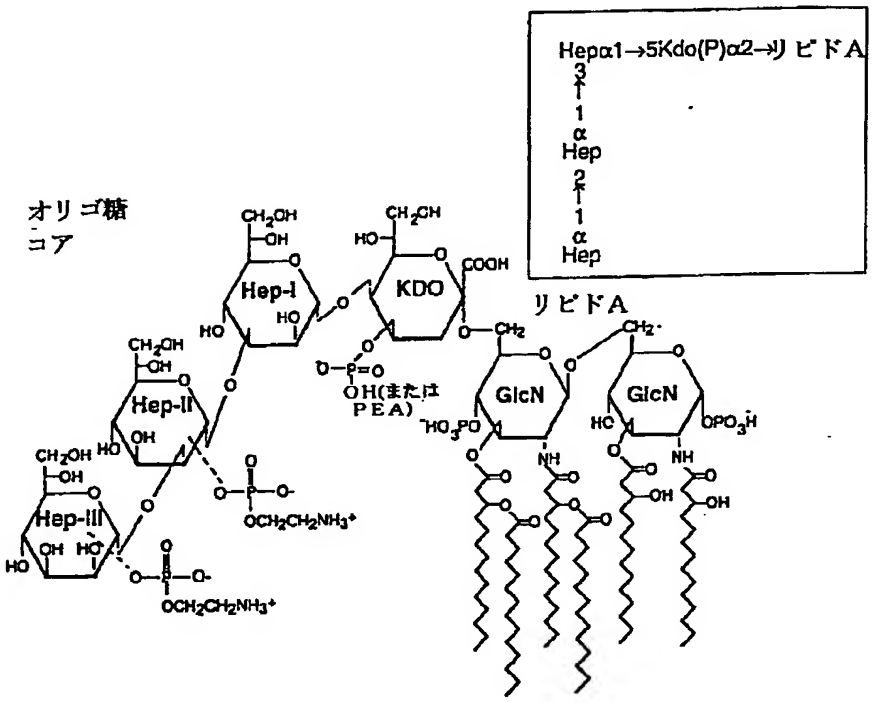
【図3】



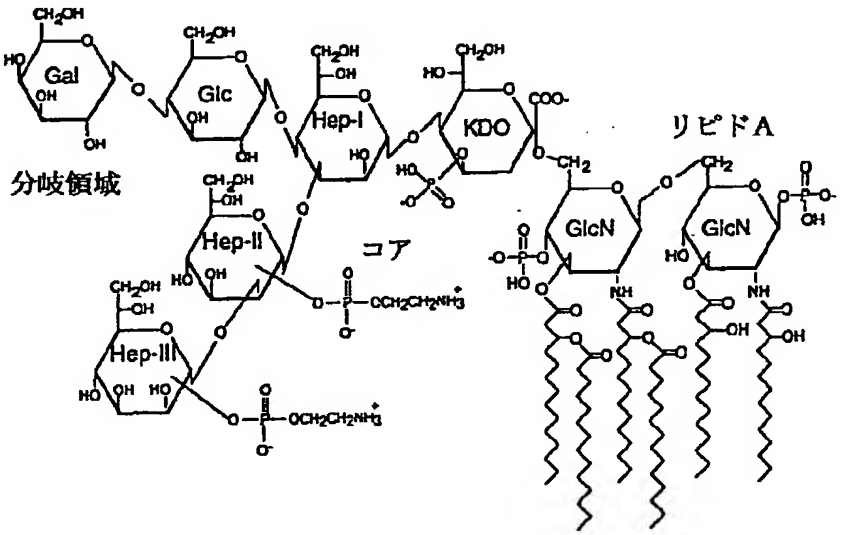
【図4】


Kdo
2
α
↓
4
Hepα1→5Kdoα2→リピドA
3
↑
1
α
Hep--PEA
オリゴ糖
コア
リピドA
KDO
KDO
Hep-I
Hep-II
GlcN
GlcN

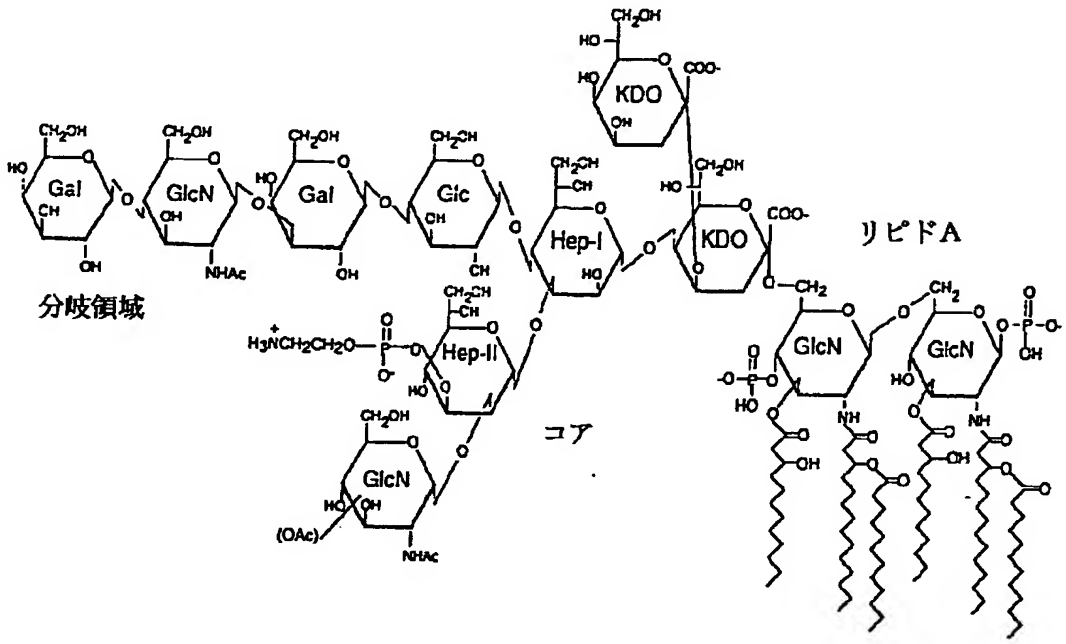
【図5】


Hepα1→5Kdo(P)α2→リピドA
3
↑
1
α
Hep
2
↑
1
α
Hep
オリゴ糖
コア
Hep-I
KDO
リピドA
Hep-II
Hep-III
OH(または
PEA)
GlcN
GlcN

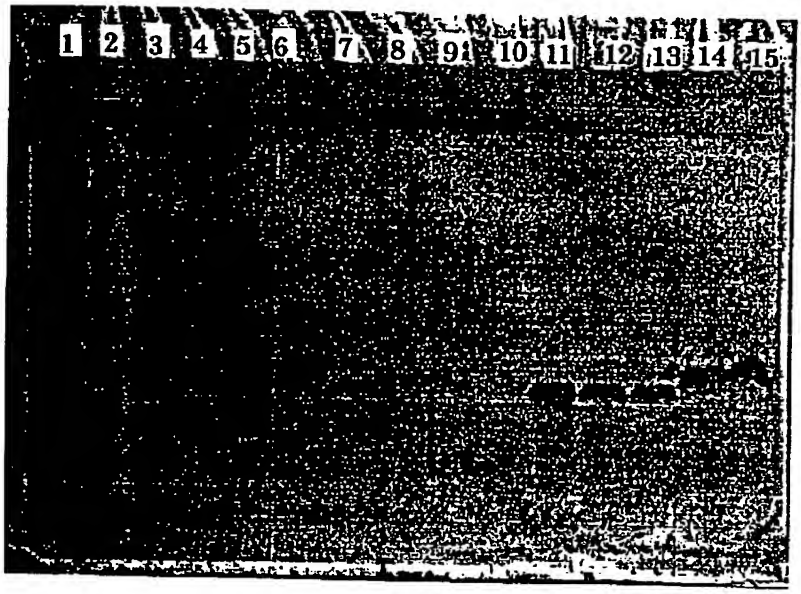
【図6】


Gal
Glc
Hep-I
KDO
リピドA
分岐領域
Hep-II
コア
Hep-III
GlcN
GlcN

【図7】


Gal
GlcN
Gal
Glc
Hep-I
KDO
KDO
リピドA
分岐領域
Hep-II
コア
GlcN
GlcN
GlcN

【図8】


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

フロントページの続き

| (51)Int.Cl.[6] | 識別記号 | FI |
|---|---|---|
| A61K | 39/10 | A61K 39/10 |
| | 39/104 | 39/104 |
| | 39/385 | 39/385 |

(72)発明者　マリア・フォーチュナーネビン
アメリカ合衆国14580ニューヨーク州ウェブスター、サミット・ドライブ696番

(72)発明者　マイケル・エイ・アピセッラ
アメリカ合衆国52333アイオワ州ソロン、ジョンソンズ・クロッシング2626番

(72)発明者　ブラッドフォード・ダブリュー・ギブソン
アメリカ合衆国94702カリフォルニア州バークレー、ベラルタ・アベニュー1324番

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/22994 A3

(51) International Patent Classification[7]: A61K 39/095, 39/40, A61P 31/04, C07K 16/12, G01N 33/68

(21) International Application Number: PCT/GB00/03758

(22) International Filing Date: 2 October 2000 (02.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 60/156,940 | 30 September 1999 (30.09.1999) | US |
| 60/196,305 | 12 April 2000 (12.04.2000) | US |

(71) Applicant *(for all designated States except US)*: **ISIS INNOVATION LIMITED** [GB/GB]; Ewert House, Ewert Place, Summertown, Oxford, Oxfordshire OX2 7BZ (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PLESTED, Joyce, Susan** [GB/GB]; Department of Clinical Immunology, Churchill Hospital, Headington, Oxford OX3 7LJ (GB). **JENNINGS, Michael, Paul** [AU/AU]; Department of Microbiology, University of Queensland, St. Lucia, Brisbane, QLD 4072 (AU). **GIDNEY, Margaret, Ann, Jaqueline** [CA/CA]; Institute for Biological Sciences, National Research Council, Ottawa K1A OR6 (CA). **COX, Andrew, David** [GB/CA]; Institute for Biological Sciences, National Research Council, Ottawa K1A OR6 (CA). **RICHARDS, James, Clare** [CA/CA]; Institute for Biological Sciences, National Research Council, Ottawa K1A OR6 (CA). **MOXON, Richard, Edward** [GB/GB]; Molecular Infectious Disease Group, Oxford University Department of Paediatrics, John Radcliffe Hospital, Oxford OX3 9DU (GB).

(74) Agent: **RUFFLES, Graham, Keith**: Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

(88) Date of publication of the international search report:
11 October 2001

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/22994 A3

(54) Title: VACCINES AGAINST *NEISSERIA* INFECTION

(57) Abstract: The invention relates to a vaccine for the treatment of disease caused by *Neisseria*, the vaccine comprising one or more immunogenic components for *Neisseria* serogroups, as well as antibodies to the immunogenic components and methods of preventing and treating *Neisseria* infections. The immunogens are based on elements of the inner core lipopolysaccharide.

# INTERNATIONAL SEARCH REPORT

Inter

Intel Application No
PCT 00/03758

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/095 A61K39/40 A61P31/04 C07K16/12 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US 5 705 161 A (VAN DER LEY P.A. ET AL.) 6 January 1998 (1998-01-06) cited in the application the whole document --- | 1-41 |
| A | ANDERSEN SVEIN RUNE ET AL: "Lipopolysaccharide heterogeneity and escape mechanisms of Neisseria meningitidis: Possible consequences for vaccine development." MICROBIAL PATHOGENESIS, vol. 23, no. 3, 1997, pages 139-155, XP002108656 ISSN: 0882-4010 the whole document --- | 1-45 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search
2 April 2001

Date of mailing of the international search report
16/05/2001

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inter[redacted]l Application No
PCT/[redacted]00/03758

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 5705161 A | 06-01-1998 | NL 9201716 A | 02-05-1994 |
| | | AU 684720 B | 08-01-1998 |
| | | AU 4835193 A | 26-04-1994 |
| | | EP 0680512 A | 08-11-1995 |
| | | FI 951535 A | 01-06-1995 |
| | | JP 8501940 T | 05-03-1996 |
| | | WO 9408021 A | 14-04-1994 |
| | | NO 951181 A | 01-06-1995 |

PATENT COOPERATION TREATY

# PCT

REC'D 25 JAN 2002

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| WPP82704 | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/GB00/03758 | 02/10/2000 | 30/09/1999 |
| International Patent Classification (IPC) or national classification and IPC<br>A61K39/095 | | |
| Applicant<br>ISIS INNOVATION LIMITED et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 26/04/2001 | 22.01.2002 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer<br>Barz, W<br>Telephone No. +49 89 2399 7320 |

Form PCT/IPEA/409 (cover sheet) (January 1994)

# INTERNATIONAL SEARCH REPORT

Inter[redacted] Application No
PCT/[redacted]00/03758

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 5705161 A | 06-01-1998 | NL 9201716 A | 02-05-1994 |
| | | AU 684720 B | 08-01-1998 |
| | | AU 4835193 A | 26-04-1994 |
| | | EP 0680512 A | 08-11-1995 |
| | | FI 951535 A | 01-06-1995 |
| | | JP 8501940 T | 05-03-1996 |
| | | WO 9408021 A | 14-04-1994 |
| | | NO 951181 A | 01-06-1995 |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03758

## I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
**Description, pages:**

1-59 as originally filed

**Claims, No.:**

1-41 as originally filed

**Drawings, sheets:**

1/15-15/15 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   *(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**II. Priority**

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   **see separate sheet**

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 36-37, 41-42 (IA).

because:

☒ the said international application, or the said claims Nos. 36-37, 41-42 (IA) relate to the following subject matter which does not require an international preliminary examination (*specify*):
**see separate sheet**

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-38, 40-41 |
| | No: | Claims | 39 |
| Inventive step (IS) | Yes: | Claims | 1-37, 40-41 |
| | No: | Claims | 38-39 |
| Industrial applicability (IA) | Yes: | Claims | 1-35, 38-39 |
| | No: | Claims | |

2. Citations and explanations
**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
**see separate sheet**

**ITEM II:**

The present application claims priority to two earlier patent applications. However, only one of said applications (filing date 30.09.99) was filed before the publication date of the two prior art documents which were listed as "P,X" documents in the International Search Report. Therefore, it appears that the priority is not validly claimed for **claims 15-19, 32, and 34** of the present application, because said priority document does not seem to disclose any other antibody than B5. Since, however, the subject-matter of said claims does not appear to be disclosed in the "P,X" documents listed in the International Search Report, said documents do not seem to be relevant for the present application.

**ITEM III:**

**Claims 36-37 and 40-41** relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT). See, however, item V-3. below.

**ITEM V:**

Reference is made to the following document:

D1: US-A-5 705 161 (Van Der Ley P.A. et al.), 6 January 1998, cited in the application.

1. NOVELTY

**Claim 39** does not meet the requirements of Article 33(2) PCT for the following reason:

1.1 The use of one or more biosynthetic pathway genes in the production of a Neisseria strain for the assessment, treatment or prevention of Neisseria infection is disclosed in document D1 (abstract; claim 8). Therefore, **claim 39** does not fulfill the requirement of novelty (Article 33(2) PCT).

1.2 The remaining **claims 1-38 and 40-41** appear to be novel, because none of the available prior art documents discloses the same combination of features as these claims.

2. INVENTIVE STEP

2.1 Document D1, which is considered to represent the most relevant state of the art, discloses a vaccine for the treatment of disease caused by pathogenic Neisseria, said vaccine comprising an immunogenic component based on the inner core of a Neisseria LPS (abstract; column 4, lines 1-47; claims 1-2 and 6). Compared to said prior art vaccine, the subject-matter of present **claim 1** differs in that the immunogenic component is capable of eliciting functional antibodies against a majority of pathogenic Neisseria strains (including serogroup B strains). Therefore, the technical problem to be solved by the present invention may be regarded as how to provide an vaccine which is effective against a majority of pathogenic Neisseria strains.

Irrespective of the objection under item VIII-1. below, the solution proposed in present claim 1 appears to involve an inventive step (Article 33(3) PCT), because the available prior art does not disclose any immunogenic component which is representative for the LPS inner core of a majority of Neisseria strains. Furthermore, the present application clearly demonstrates the conservation of said immunogenic component across the diversity of meningococcal disease isolates and the accessibility of said immunogenic component in encapsulated strains (see Example 1). Since the application also provides evidence for the bactericidal activity of the B5 antibody (see Example 3), the present application appears to be the first disclosure of an effective vaccine against a majority of pathogenic Neisseria strains. Consequently, claim 1 seems to be inventive in the sense of Article 33(3) PCT.

2.2 **Claim 15** also appear to involve an inventive step (Article 33(3) PCT) for analogous reasons as outlined in item V-2.1 above.

2.3 **Claims 2-14 and 16-28** are dependent on claim 1 and/or claim 15 and as such also meet the requirements of the PCT with respect to inventive step (Article 33(3) PCT).

2.4 **Claims 29-32** relate to antibodies reactive with the immunogenic component defined in claims 1-28. Similarly, **claims 33-35** relate to hybridomas producing and pharmaceutical preparations comprising such antibodies. Due to the arguments provided in item V-2.1 above, an inventive step can also be acknowledged for said claims.

2.5 **Claims 36-37** relate to methods for treating Neisseria infection comprising administering the inventive vaccine of claims 1-28 or the inventive antibody of claims 29-31. Therefore, said claims also appear to meet the requirements of the PCT with respect to inventive step (Article 33(3) PCT).

2.6 In contrast, the identification method of **claim 38** does not appear to involve an inventive step (Article 33(3) PCT), because said method comprises only steps which fall within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. Consequently, the skilled person would regard it a normal design procedure to combine all the steps set out in claim 38. Thus, the subject-matter of claim 38 does not involve an inventive step and does not satisfy the criterion set forth in Article 33(3) PCT.

2.7 The second medical use **claims 40-41** appear to involve an inventive step (Article 33(3) PCT), because they relate to the use of the inventive immunogenic component or antibody (see above).

3. INDUSTRIAL APPLICABILITY

For the assessment of the present **claims 36-37 and 40-41** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

**ITEM VIII:**

1. **Claims 1 and 15** are neither disclosed in a manner sufficiently clear and complete (Article 5 PCT) nor fully supported by the description (Article 6 PCT). The reasons therefor are that said claims are broader than justified by the description and that they attempt to define the subject-matter in terms of the result to be achieved ("immunogenic component capable of eliciting functional antibodies [...]") which merely amounts to a statement of the underlying problem.

2. The term "majority" used in **claims 1 and 15** is vague and unclear and leaves the reader in doubt as to the percentage to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

3. Similarly, the expression "functional equivalent thereof" used in **claims 9-10** is not clear (Article 6 PCT) and leaves the reader in doubt as to the technical features of said "functional equivalent".

4. The expression "a few immunogenic components" in **claim 15** is also not clear, because leaves the reader in doubt concerning the precise number of such components.

5. Finally, **claims 33-34** are not clear in the sense of Article 6 PCT, because the antibody nomenclature "B5" and "A4" appears to be an internal nomenclature which is not generally known.